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Exposure and response of human non-neuronal cells to prions *in vitro*

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DECLARATION

I declare that the work presented herein is my original research and that I wrote this thesis. The experiments were designed by myself under the supervision of my primary PhD supervisor, Dr Mark Head. Where contributions were made by others, their involvement is described in the Acknowledgements and in the thesis. The enclosed publication was drafted by my PhD supervisor, Dr Mark Head in collaboration with the other co-authors, including myself, and my contribution to the paper's presentation and content was significant.

No part of this work has been or will be submitted for any other degree of professional qualification.

Zuzana Krejciova

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ABSTRACT

Despite intensive research, the cellular and molecular mechanisms involved in human cellular susceptibility to prion infection remain poorly defined, in part due to the continuing lack of cultured human cells that are susceptible to infection with human prions. Such culture models would present distinct advantages including speed and expense compared with animal models, and would provide systems in which to investigate the interaction between PrP^{C} and PrP^{Sc}, the basis of cellular susceptibility, the nature of the species barrier and the mechanism of prion propagation *in situ*.

This study sought to examine whether non-neuronal cells might provide opportunities to establish human cell lines replicating human prions. A human follicular dendritic cell-like cell line (termed HK) was obtained, further characterised and then tested for its ability to support human prion replication. The mechanisms of internalisation, intracellular trafficking and the eventual fate of exogenous PrP^{Sc} taken up by these cells were also examined. This thesis similarly examined the cellular response of human embryonic stem cells (hESC) to acute exposure to human and animal prions.

PrP^{C} was found to be abundantly expressed by HK cells and HK cell extracts were found to support conversion to PrP^{Sc} in a cell-free conversion assay. However, HK cells exposed to infectious brain homogenates failed to accumulate PrP^{Sc} or become infected *in vitro*. Exposed HK and hESC did display a readily detectable, time dependent uptake of PrP^{Sc} from medium spiked with prion-infected brain homogenates that was independent of the species, disease phenotype and *PRNP*

codon 129 genotype of the human source and the recipient cells. The exposed cells showed intensely labelled intracellular accumulations of PrP^{Sc} with coarse granular morphology, largely in the juxtanuclear region of cytoplasm. However, when the brain-spiked medium was withdrawn and cells were given control medium, the intensity and extent of PrP^{Sc} immunostaining rapidly diminished. Co-localisation studies implicated caveolae-mediated endocytic uptake of exogenous PrP^{Sc}, apparently preceding uptake via clathrin coated pits in HK cells. Evidence suggesting that the endosomal recycling compartment and lysosomes are involved in intracellular trafficking and degradation of exogenous PrP^{Sc} was also found.

Understanding the cell biology of these processes may help to explain why the majority of cultured cells are refractory to prion infection *in vitro*. Internalization of misfolded PrP and its subsequent degradation in the lysosomal compartment might function as a self-protective cellular mechanism, serving to eliminate non-native, presumably dysfunctional and potentially dangerous PrP conformers, whether generated endogenously or acquired through exposure to exogenous prion infectivity.

LIST OF ABBREVIATIONS

ACDP	Advisory Committee on Dangerous Pathogens
AD	Alzheimer's disease
ADAM/TACE	a disintegrin and metalloprotease/TNF α-converting enzyme
ATB	antibiotics
BASE	bovine amyloidotic spongiform encephalopathy
Bax	B-cell lymphoma 2-associated X protein
Bcl-2	B-cell lymphoma 2
bp	base pair
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
C	cytosine
C-	end of peptide chain carrying the free alpha-carboxyl group (also COOH-)
hop/STI1	co-chaperone adaptor protein for Hsp70/Hsp90 complexes
LN	laminin
CHO	carboxyhydrate
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CO_2	carbon dioxyde
CSF	cerebrospinal fluid
^{Ctm}PrP	transmembrane PrP with an extracellular C-terminus
CWD	chronic wasting disease
D'PBS	Dulbecco's PBS
DAPI	4,6'-diamino-2-phenyl indole
DC	dendritic cells
dH_2O	distilled water
DNA	deoxyribonucleic acid
Dpl	doppel
DRM	detergent-resistant membrane
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
EEG	electroencephalography
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
EUE	exotic ruminant encephalopathy
EVB	Epstein-Barr virus
fCJD	familial Creutzfeldt-Jakob disease
FCS	foetal calf serum
FDC	follicular dendritic cells
FFI	familial fatal insomnia
FITC	fluorescein isothiocyanate
FSE	feline spongiform encephalopathy
G	guanine
GALT	gut-associated lymphatic tract
GFAP	glial fibrillary acidic protein

GFP	green fluorescent protein
Gnd	guanidinium
GPI	glycosylphosphatidylinositol
GPI-AP	glycosylphosphatidylinositol-anchored protein
GSS	Gerstmann-Sträussler-Schienker disease
-GTG-	guanine-thymine-guanine
GTP	guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HC	hydrophobic region
hESC	human embryonic stem cells
HK	the HK cell line established by **H**an-Soo **K**im
Hu MM Tg	humanised transgenic mice homozygous for methionine
ICC	immunocytochemistry
iCJD	iatrogenic Creutzfeldt-Jakob disease
IgG	immunoglobuline G
kb	kilo base pairs
kDa	kilo Dalton
LAMP1	lysosome associated membrane glycoprotein 1
LAMP2b	lysosome associated membrane glycoprotein 2b
LRP/LR	laminin receptor precursor/laminin receptor
M	molar
mAb	monoclonal antibody
MeOH	methyl alcohol
MM	methionine homozygous at *PRNP* codon 129
MM1	MM1 subtype of CJD
MRC	Medical Research Council
MRHA	Medicines and Healthcare products Regulatory Agency
mRNA	messenger ribonucleic acid
MV	methionine/valine heterozygous at *PRNP* codon 129
N-	amino terminal end of the peptide chain (also NH_2-)
NaCl	sodium chloride
NANOG	key factor in maintaining embryonic stem cells pluripotency
NaOH	sodium hydroxide
NCAM	neuronal cell adhesion molecule
NCJDRSU	National CJD Research & Surveillance Unit
^{Ntm}PrP	transmembrane PrP with an extracellular N-terminus
NuPAGE LDS	sample buffer for polyacrylamide gel electrophoresis containing lithium dodecyl sulfate at a pH of 8.4
ORF	open reading frame
p	cell passage number
p53	tumor protein 53
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.1% Tween 20
PCR	polymerase chain reaction
PFA	paraformaldehyde
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PNS	peripheral nervous system
Prnd	mouse doppel protein gene
PRNP	gene encoding prion protein in humans and sheep

Prnp	gene encoding prion protein in other non-human species
PrP	prion protein, product of the prion protein gene
$PrP^{27\text{-}30}$	truncated proteinase K-resistant core of PrP^{Sc}
PrP^{C}	normal cellular form of the prion protein
PrP^{L}	intermediate or toxic side product produced during PrP^{C} to PrP^{Sc} conversion
PrP^{res}	partially protease-resistant form of PrP^{Sc}
PrP^{Sc}	abnormal 'scrapie'form of the normal prion protein
PVDF	polyvinylene difluoride
QuIC	quaking-induced conversion
rab11A	member of Ras superfamily of monomeric G proteins
RCM-1	Roslin Cells Manchester-1
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-QuIC	real-time quaking-induced conversion
ryb/son/filt	ribolysed/sonicated/filtered
SCA	scrapie cell assay
sCJD	sporadic Creutzfeldt-Jakob disease
SDS	sodium dodecyl sulphate
SDS-PGE	sodium dodecyl sulphate polyacryamide gel electrophoresis
sFI	sporadic fatal insomnia
S-S	disulfide bond
SYBR	asymetrical cyanine dye used as a nucleic acid stain
TBS-T	tris-buffered saline with 0.1% Tween 20
TC	temporal cortex
TME	transmissible mink encephalopathy
TNT	tunnelling nanotubes
Tris-HCL	tris-hydrochloride
TSE	transmissible spongiform encephalopathy
UK	United Kingdom
UV	ultra violet
vCJD	variant Creutzfeldt-Jakob disease
VPSPr	variably protease-sensitive prionopathy
VV	valine homozygous at *PRNP* codon 129
VV2	VV2 subtype of CJD
w/v	weight per volume
WHO	World Health Organisation

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1. INTRODUCTION

1.1 PRIONS – OVERVIEW

The concept of prion diseases originated in the field of transmissible spongiform encephalopathies (TSEs), which are invariably fatal neurodegenerative disorders of the central nervous system, naturally occurring in humans and a wide range of animals. The term "spongiform" relates to the neuropathological alteration of brain tissue (sponge-like vacuolation in the grey matter) in affected individuals. Prions are a novel class of infectious pathogens in which a misfolded host-encoded protein appears to be the main component of the disease-causing agents. The normal cellular prion protein (PrP^{C}) was first discovered in the context of its pathogenic "scrapie" isoform (PrP^{Sc}), which plays a key role in the development of TSEs.

The nature of the infectious agents responsible for TSEs has been a matter of debate for many years. The prototypic prion disease scrapie, which naturally occurs in sheep and goats, was recognised in Europe over 200 years ago (McGowan, 1922). Its transmissibility was first demonstrated by J. Cuille and P. Chelle in 1936 who performed intraocular inoculation of sheep with infected brain (Cuille and Chelle, 1936). In 1954 the concept of "slow virus" infection was proposed by B. Sigurdsson, which included "rida" in sheep (the Icelandic name for the scrapie prion infection) (Sigurdsson, 1954). The transmissibility of scrapie to other mammalian species (goats, rats, mice, and hamsters) was also demonstrated (Cuille and Chelle, 1939; Chandler, 1963; Chandler and Fisher, 1963; Zlotnik, 1963) and the long incubation period was considered to result from a "species barrier" (Pattison, 1965). Unlike other infectious diseases of bacterial, viral, or viroid origin, the pathogen appeared to

be resistant to inactivation by UV and ionizing radiation, but was affected by chaotropic agents (Alper *et al.*, 1966; Marsh *et al.*, 1974; Ward *et al.*, 1974; Alper *et al.*, 1978; Diener *et al.*, 1982), thus indicating an infectious agent devoid of nucleic acid. A protein as a pathological agent was proposed in 1967 by I. Pattison and K. Jones (Pattison and Jones, 1967) and the existence of more than one strain of scrapie and a model for the scrapie agent self-replication was proposed by the mathematician J. Griffith (Griffith, 1967). However, these proposals were met with scepticism and were relatively neglected by the scientific community as they challenged the central dogma of molecular biology, that genetic information flows from DNA to RNA to protein. In 1982, the physician and scientist of the Johns Hopkins University School of Medicine, R. Johnson summarised the situation: *"The unorthodox properties of the spongiform encephalopathy agents have created a mystique about their possible nature. Exotic fantasies have evolved of an agent devoid on nucleic acids but representing self-replicating membranes, proteins or polysaccharides."* (Johnson, 1982).

In the 1920s, the two sets of neurological cases reported independently by H. Creutzfeldt and A. Jakob (Creutzfeldt, 1920; Jakob, 1921) were given the name Creutzfeldt-Jakob disease (CJD) by W. Spielmeyer in 1922. In 1936 the initial description of Gerstmann-Sträussler-Schienker disease (GSS) suggested that this was an inherited disease rather than acquired by transmission despite its characteristics of spinocerebellar ataxia with a distinctive neuropathological phenotype (Gerstmann *et al.*, 1936; Gajdusek and Zigas, 1957; Gajdusek and Zigas, 1959). In the 1950s C. Gajdusek was attracted to the highlands of Papua New Guinea by reports of a mysterious epidemic, primarily afflicting women and children called kuru that was

ravaging the native tribes of the Fore people (Gajdusek and Zigas, 1957; Gajdusek and Zigas, 1959). Kuru was characterised by progressive ataxia and neurodegeneration. In 1959 W. Hadlow pointed out the striking similarity between scrapie and kuru at their neuropathological, clinical and epidemiological level. This lead to the hypothesis that kuru could be a transmissible disease (Hadlow, 1959) and specifically that it was transmitted by ritual cannibalism (Gajdusek, 1977). Kuru was successfully transmitted to chimpanzees in 1966 (Gajdusek *et al.*, 1966), and this was followed by the successful transmissions of sporadic CJD in 1968 (Gibbs, Jr. *et al.*, 1968) and GSS in 1981 (Masters *et al.*, 1981). However, it is important to note that not all forms of GSS were successfully transmitted (Brown *et al.*, 1994a; Tateishi and Kitamoto, 1995; Parchi *et al.*, 1998). These pioneering studies established that kuru and CJD, which were considered to be diseases of unknown etiology, belonged to the same group of diseases. Gajdusek was awarded by the Nobel Prize in 1976 for his work on kuru.

The term "prion" was coined by a Nobel Laureate, S. Prusiner in 1982 as an acronym denoting "small ***pro***teinaceous ***in***fectious particle that resists inactivation by procedures which modify nucleic acids" (Prusiner, 1982). Additional investigations demonstrated further anomalies peculiar to prion diseases. Most prominent is the absolute requirement for the presence of the normal ***c***ellular prion protein, PrP^{C} (Bueler *et al.*, 1993), and interplay with the pathogenic protease resistant disease associated conformer of this prion protein, termed PrP^{Sc} after the first described prion disease, ***sc***rapie, in sheep. In 1982, a fragment of the prion protein termed $PrP^{27\text{-}30}$ was discovered and further molecular cloning studies of the prion protein gene revealed that the $PrP^{27\text{-}30}$ was the truncated proteinase K-resistant core of the disease-

associated prion protein, PrP^{Sc}, which had the same amino acid sequence as the normal cellular prion protein, PrP^{C} (Bolton *et al.*, 1982; Prusiner *et al.*, 1982; Chesebro *et al.*, 1985; Oesch *et al.*, 1985; Hope *et al.*, 1986; Meyer *et al.*, 1986) and was shown to retain infectivity (Riesner, 2003). Prion protein was shown to be encoded by a chromosomal gene and not by the nucleic acid of an infectious scrapie particle. Levels of PrP mRNA were observed to remain unchanged throughout the course of scrapie infection (Basler *et al.*, 1986). PrP^{Sc} was therefore suggested to be a misfolded, disease associated isoform derived from host's own PrP^{C}, which is an abundantly expressed glycosylphosphatidylinositol- (GPI-) anchored plasma membrane protein (Hope and Manson, 1991; Prusiner, 1998). In 1998 Prusiner proposed the "protein only" hypothesis of prion replication in which he states that an abnormal isoform, PrP^{Sc}, of the normal cellular prion protein, PrP^{C}, is capable of recruiting the PrP^{C} to undergo a change of conformation into PrP^{Sc} without the presence of DNA (or RNA) (Prusiner, 1998). These key observations have allowed the identification of a wide spectrum of human and animal prion diseases (Figure 1.1) (Ironside, 1996; Ironside, 1998).

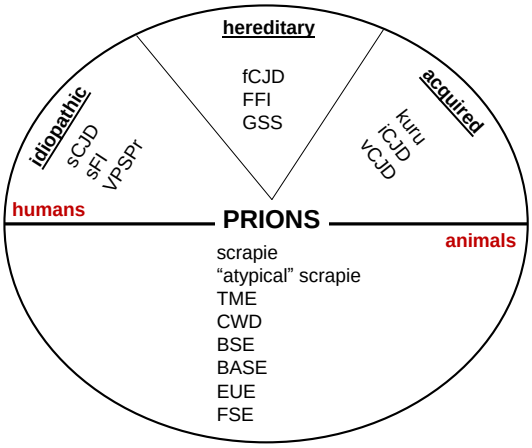


Figure 1.1: Classification of prion diseases of humans and animals
Human prion diseases can be classified and further sub-classified as idiopathic: sporadic Creutzfeldt-Jakob disease (sCJD), sporadic fatal insomnia (sFI), variably protease-sensitive prionopathy (VPSPr); hereditary: familial Creutzfeldt-Jakob disease (fCJD), familial fatal insomnia (FFI), Gerstmann-Sträussler-Schienker disease (GSS); and acquired: kuru, iatrogenic Creutzfeldt-Jakob disease (iCJD), variant Creutzfeldt-Jakob disease (vCJD). Animal prion diseases are sub-classsified as: scrapie, atypical scrapie, transmissible mink encephalopathy (TME), chronic wasting disease (CWD), bovine spongiform encephalopathy (BSE), bovine amyloidotic spongiform encephalopathy (BASE), exotic ruminant encephalopathy (EUE), feline spongiform encephalopathy (FSE).

Since the Central Veterinary Laboratory in England reported the first case of a novel prion disease in cattle, termed bovine spongiform encephalopathy (BSE) in 1987, TSEs have attracted a broad interest (Wells *et al.*, 1987; Parchi *et al.*, 1997). This prion disease was transmitted via livestock feed supplemented with bone and meat meal derived from unrecognised prion-infected animal sources, most likely from scrapie infected sheep. BSE was shown to be transmissible, highly pathogenic and retaining its biological identity after crossing between species either naturally or experimentally (Bruce *et al.*, 1994; Bruce *et al.*, 1997). In 1996, yet another novel TSE was discovered, this time in humans, which was termed new variant CJD or vCJD (Will *et al.*, 1996). Histopathological, biochemical, and epidemiological evidence suggests that vCJD is most likely to be a result of orally ingested infectious

BSE prion agent and is believed to have caused over 200 cases of vCJD worldwide (Chazot *et al.*, 1996; Collinge *et al.*, 1996; Will, 1996; Hill *et al.*, 1997a; Prusiner *et al.*, 1998) (http://www.cjd.ed.ac.uk/vcjdworld.htm). Moreover, four cases of vCJD infection have been reported to be caused by blood transfusion (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006; Peden *et al.*, 2010), in which prions were transmitted by from patients in the pre-clinical phase of the disease.

The majority of the recent clinical applications involving human stem cell therapies are still critically dependent in their development on poorly characterised human and animal bioproducts as well as isolating blood products for therapies (Reubinoff *et al.*, 2000; Lanzendorf *et al.*, 2001; Mitalipova *et al.*, 2003; Hovatta *et al.*, 2003; Cowan *et al.*, 2004; Klimanskaya *et al.*, 2005; Fletcher *et al.*, 2006; Ludwig *et al.*, 2006). The potential for inadvertent prion transmission in humans by emerging cellular therapies has received comparatively little attention from the public health point of view (Cobo *et al.*, 2006; Akimov *et al.*, 2008; Akimov *et al.*, 2009; Cushman *et al.*, 2010). The main prion protein characteristics and spectrum of prion diseases related to this project are described in more detail in the sections that follow.

1.2 THE PRION PROTEIN

The prion protein (PrP) is a fundamental prerequisite for all prion diseases and is expressed in a variety of different vertebrate classes including fish, reptiles, birds, and mammals. This PhD project employed mammalian (human) cell culture systems and involved the use of human and bovine prions. Here, PrP always refers to the mammalian prion protein. Some of the cellular processes mentioned in this thesis may also correlate with prion protein processes in other vertebrates. Interestingly,

there is an obvious sequence homology between some parts of mammalian and non-mammalian prion proteins, indicating that many of the important structural and functional features are most likely conserved (Calzolai *et al.*, 2005; Lysek *et al.*, 2005; Rivera-Milla *et al.*, 2006; Ji and Zhang, 2007). In this thesis, the terms used for the different prion protein forms are stated in Table 1.1, except where citing the work of others, when terminology used as in the original publication is adopted. It is also important to note the terminology used for prion protein in the experimental part of this thesis when referring to the pre-treatment procedure used in the visualisation process (for example, when pre-treatment of cells with proteinase K and/or guanidine thiocyanate is used).

Term	Definition
PRNP	Gene encoding prion protein in humans and sheep
Prnp	Gene encoding prion protein in other species
PrP	Prion protein, product of the prion protein gene
PrP^{C}	Normal cellular form of the prion protein
PrP^{Sc}	Abnormal prion disease-associated form of the prion protein, denoted after scrapie, also visualised by confocal microscopy after incorporating the proteinase K and guanidine pre-treatment in the immunocytochemical procedure of cells exposed to prion infected brain homogenate
PrP^{res}	Partially protease-resistant form of the prion protein, seen in biochemical analysis (Western blot) after proteinase K digestion, not necessarily synonymous with PrP^{Sc}
Exogenous prion protein	The form of the prion protein detected by confocal microscopy in cells exposed to infectious prion infected brain homogenates. When proteinase K or guanidine pre-treatment was not included in the immunocytochemical procedure four forms of prion protein could be detected: brain derived PrP^{Sc} and PrP^{C}, endogenous PrP^{C} of exposed cells and PrP^{Sc} when assuming the prion infection of the exposed cells

Table 1.1: Terms used for the different prion protein forms

1.2.1 The prion protein gene

The human prion protein gene was first described in 1986 (Liao *et al.*, 1986) and the complete DNA sequence is available at the Internet bioinformatics site (GenBank, accession number AL133396). In humans, the prion protein gene (*PRNP*) is a single copy gene, located on the short arm of chromosome 20 and is composed of two exons and a single intron that span about 15 kb (Figure 1.2). The entire open reading

frame (ORF) containing the complete 762 bp long DNA sequence encoding the 253 amino acid residues of human prion protein is located within the exon 2 (Sparkes *et al.*, 1986; Prusiner, 1991a). *PRNP* transcription is controlled via a promoter region located upstream of exon 1 and contains a single major transcription start site (Puckett *et al.*, 1991) unusually rich in G, C nucleotides. Those within the mRNA transcript could potentially be involved in the secondary structure formation if bound to the GC rich region of the open reading frame (Goldmann, 1993).

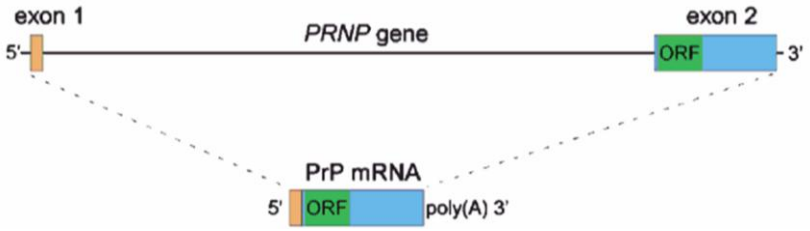


Figure 1.2: Diagrammatic representation of *PRNP* and PrP mRNA
The *PRNP* open reading frame ORF (green) is located within exon 2. Figure source (Kong and Bessen, 2008).

The prion protein gene is often classified as a housekeeping gene, based on the absence of a TATA box in the prion promoter. However, there are highly conserved motifs for binding transcription factors and a consensus heat shock factor binding site present in the immediate 5' region (Basler *et al.*, 1986; Puckett *et al.*, 1991; Westaway *et al.*, 1994; Saeki *et al.*, 1996; Baybutt and Manson, 1997; Inoue *et al.*, 1997; Shyu *et al.*, 2002). Expressions of both PrP^{C} mRNA and protein are developmentally regulated, increasing postnatally with distinct time course for specific regions as observed in rat, hamster and mouse brains (Lieberburg, 1987; McKinley *et al.*, 1987; Lazarini *et al.*, 1991; Manson *et al.*, 1992). Induction of *Prnp* mRNA expression was first detectable around the ninth embryonic day of mouse, coinciding with the transition from anaerobic to aerobic metabolism (Miele *et al.*,

2003). The genes encoding prion protein of various species were also sequenced in 1986 (Basler *et al.*, 1986; Liao *et al.*, 1986; Locht *et al.*, 1986). Most animals including cattle, sheep and mice have their prion protein gene composed of three exons with the ORF within exon 3, which is analogous to exon 2 of the human prion protein gene (Prusiner, 1998). The prion protein gene was shown to be highly conserved across many animal species, suggesting functional importance for the prion protein (Lee *et al.*, 1998; Van Rheede *et al.*, 2003; Premzl and Gamulin, 2007). DNA variations of the human prion protein gene fall into two categories: mutations that are associated with a prion disease and are highly penetrant, and polymorphisms that do not themselves directly result in disease, but which can have an influence on susceptibility to prion infection or a profound effect on the clinical features of the resultant disease (Figure 1.3) (Palmer *et al.*, 1991; Collinge *et al.*, 1991b; Goldfarb *et al.*, 1992; Windl *et al.*, 1996; Zeidler *et al.*, 1997; Parchi *et al.*, 1999a). More than thirty pathogenic mutations including point mutations resulting in amino-acid substitution, deletion or insertion of additional octapeptide repeats have been described to date (Kong *et al.*, 2004; Mead *et al.*, 2006). These are linked to, or have been associated with specific familial prion diseases phenotypes (reviewed Kong *et al.*, 2003). From the over twenty natural *PRNP* polymorphisms, only the polymorphism at codon 129 (encoding either methionine or valine) has been proposed to play a role in human disease susceptibility or have an effect on disease phenotype (Collinge *et al.*, 1991a; Goldfarb *et al.*, 1992; Monari *et al.*, 1994; Parchi *et al.*, 1996; Zeidler *et al.*, 1997; Deslys *et al.*, 1998; Lee *et al.*, 2001; Brandel *et al.*, 2003).

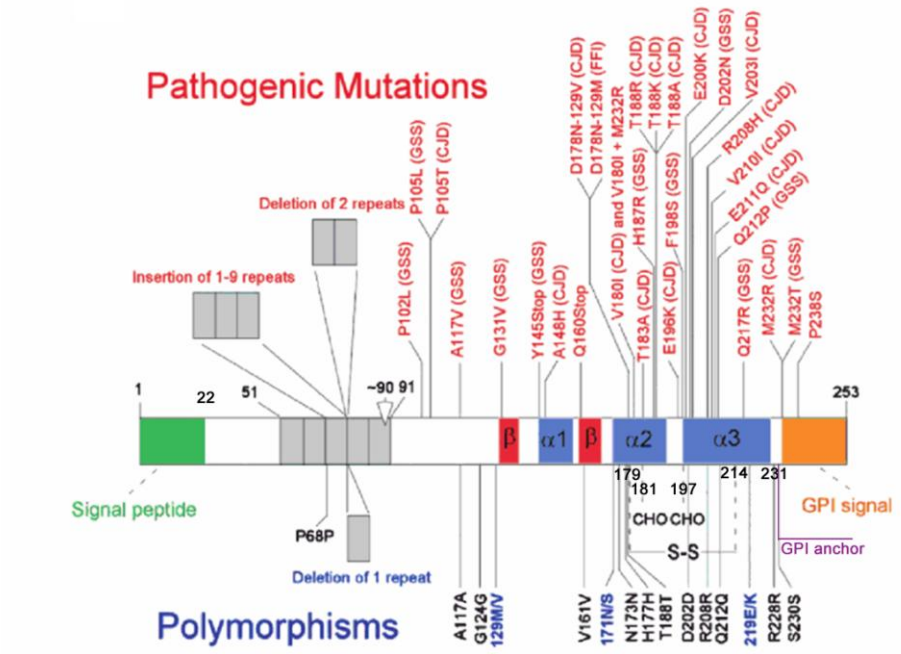


Figure 1.3: Diagrammatic representation of the primary prion protein structure including post-translational modifications, polymorphisms and pathogenic mutations
The numbers indicate amino acid residue position in the prion protein. Single letter designation for respective amino acids is used to denote polymorphisms (blue and black type) and mutations linked to the human prion diseases are indicated above (red type). The mature form of human PrP^{C} contains 208 amino acids (23-230). The secretory signal peptide (1-22) is indicated at the extreme of the N-terminus (green). The hydrophobic peptide region (231-253) (orange) is cleaved when the GPI anchor is attached (purple). The octapeptide repeats are indicated at amino acid positions 51-91 (grey boxes). The regions forming three α-helixes and two β-sheets are indicated by blue and red boxes respectively. The arrowhead at amino acid 90 indicates the major cleavage site for proteinase K. The two potential sites for N-linked glycosylation (CHO) are illustrated at residues 181 and 197 and one disulfide bond (S-S) connecting the helixes 2 and 3 at cysteine residues 179-214 is also shown. Figure modified from (Kong and Bessen, 2008).

Additional studies have suggested that the *PRNP* codon 129 genotype also may have an influence in survival (Alperovitch *et al.*, 1999; Pocchiari *et al.*, 2004), the neuropathology of sCJD (Parchi *et al.*, 1999b; Kovacs *et al.*, 2000), protease cleavage of PrP^{Sc} (Parchi *et al.*, 2000), PrP^{Sc} amyloid formation (Baskakov *et al.*, 2005) and oligomerisation of PrP^{Sc} (Lewis *et al.*, 2006). The most dramatic influence of the polymorphism at codon 129 so far observed is in its effect in vCJD (Brandel *et*

al., 2009). All genetically tested and pathologically confirmed cases of vCJD have been determined to be homozygous for methionine at this polymorphism. However, three individuals with methionine/valine (MV) genotype at the codon 129 were recently reported: one as a possible vCJD case (based on the clinical features and tests) as post-mortem examination was not performed (Kaski *et al.*, 2011) and two pre-clinical vCJD infection where one individual received a blood transfusion and one haemophiliac individual received blood products from donors incubating vCJD (Peden *et al.*, 2004; Peden *et al.*, 2010). Interestingly, these patients died without any sign of neurological symptoms. Nonetheless, the accumulation of PrP^{Sc} in the spleen was confirmed in post-mortem examination. In addition to vCJD, the association of codon 129 genotype and disease is also observed in the genotype frequencies of the sCJD (Table 1.2), in which homozygosity (either MM or VV) is more commonly associated with the disease (Brown *et al.*, 1992; Parchi *et al.*, 1999b; Head *et al.*, 2004a; Head *et al.*, 2004b; Bishop *et al.*, 2009). The one divergence from this tendency is within the UK cases of growth hormone transmitted iCJD, in which MM homozygotes represent the smallest proportion of patients (Brandel *et al.*, 2003).

***PRNP* codon 129 genotype**	**MM (%)**	**MV (%)**	**VV (%)**	**References**
Normal population (UK)	44	44.5	11.5	Bishop *et al.*, 2009
sCJD (UK)	62	19	19	www.cjd.ed.ac.uk, Annual Report 2010 (Jan MacKenzie – study co-ordinator)
vCJD (UK)	100	0	0	Brandel *et al.*, 2009
iCJD (UK) *	11	57	32	www.cjd.ed.ac.uk, Annual Report 2010 (Jan MacKenzie – study co-ordinator)

Table 1.2: Comparison of *PRNP* codon 129 frequencies within the normal population and CJD affected patients in UK
* 51 % iCJD cases were available with codon 129 genotype information

It is important to note that codon 129 occupies the second position of the four amino acids responsible for creating the β-sheet 1 and therefore may affect elements of the

tertiary structure which could be important when PrP^{C} is being recruited into PrP^{Sc} conversion (Petchanikow *et al.*, 2001). Methionine homozygosity at codon 129 was shown to be conserved within many mammalian species: cattle, sheep, rat, mice (Jew and Schatzl, 2005).

The effect of *Prnp* polymorphisms on the infection was also observed in animals. In mice the codons 108 and 189 are known to be associated with progression of the disease (Westaway *et al.*, 1994; Moore *et al.*, 1998) and in sheep three polymorphic sites of the codons 136, 154 and 171 have a profound effect on the susceptibility and pathogenesis (Hunter, 2003; Hunter, 2007).

1.2.2 The normal cellular form of the prion protein, PrP^{C}

The post-translational processing of the *PRNP* product consists of the removal of an amino terminal signal peptide consisting of 22 amino acid residues serving as an endoplasmic reticulum signal peptide, allowing insertion of the nascent PrP^{C} into the secretory pathway during synthesis. Another step of post-translational processing is removal of the carboxy terminal hydrophobic peptide formed by 23 amino acids residues and the subsequent covalent addition of the GPI moiety to serine at amino acid 231. The GPI anchor mediates prion protein attachment to the cell surface (Stahl *et al.*, 1987; Prusiner, 1991b). PrP^{C} is a monomeric protein occurring as a mixture of three major forms: un-glycosylated, mono-glycosylated and di-glycosylated, due to highly branched carbohydrate moieties attached at asparagine residues 181 and 197 (Haraguchi *et al.*, 1989). The physiological significance of PrP^{C} glycosylation is unclear. Nevertheless, protein glycosylation affects other protein properties, such as intracellular trafficking and ligand binding (Stanley, 1987; Paulson, 1989; Lis and

Sharon, 1993)The stability of PrP^C was also suggested to be modulated by N-glycans, however the experimental evidence is still lacking (Hornemann *et al.*, 2004). Interestingly, the N-terminal region of PrP^C is unstructured, while the globular C-terminal part consists of three α-helical and two very short anti-parallel β-plated sheets regions (Pan *et al.*, 1993; Riek *et al.*, 1996; Riek *et al.*, 1998; Govaerts *et al.*, 2004).

The early computational analysis of circular dichorism and low-resolution infrared spectroscopy studies indicated the high α-helical (40% of the protein) and the relatively low β-sheet content (3% of the protein) (Bazan *et al.*, 1987; Pan *et al.*, 1993; Huang *et al.*, 1994). The use of nuclear magnetic resonance spectroscopy at acid pH (Riek *et al.*, 1996; Prusiner, 1998; Hornemann *et al.*, 2004) and later crystallography (Knaus *et al.*, 2001; Haire *et al.*, 2004) refined the structural properties of PrP^C. It was found that the long flexible NH_2-proximal half of the molecule is unstructured, whereas the globular COOH-proximal half forms three α-helices corresponding, for the human PrP^C, to the residues 144-154, 173-194 and 200-228, interspersed with an antiparallel β-pleated sheets formed by β-strand at residues 128-131 and 161-164 (Figure 1.4). The structurally less defined N-terminal region consists of residues 23-124 (N-terminus to the β-sheet 1) and contains a stretch of several the octapeptide repeat sequences (Hornshaw *et al.*, 1995; Flechsig *et al.*, 2000) which are 24 bp encoding the same eight amino acids, with five repeats present in the human sequence that has been shown to bind metal ions. These are flanked by two positively charged clusters, CC1 (amino acids 23-27) and CC2 (amino acids 95-110). The hydrophobic region (amino acids 111-134) between these two domains is also known as the HC region. The structure is stabilised by

connecting the helixes 2 and 3 by a single disulfide bond occurring between cysteine residues 179 and 214 (Turk *et al.*, 1988; Riek *et al.*, 1996; Riek *et al.*, 1997; Zahn *et al.*, 2000).

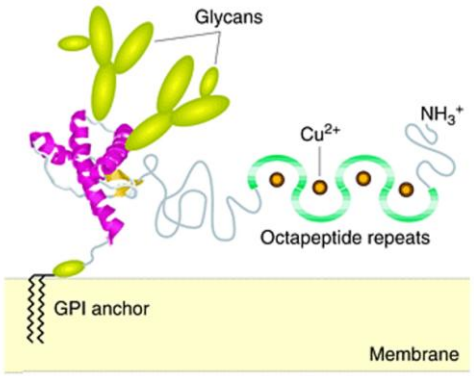


Figure 1.4: Structural features of the tertiary structure of the cellular prion protein
The cellular prion protein is anchored into a lipid bilayer. The N-terminal part (grey) is, as deduced from NMR spectroscopy, unstructured. The octapeptide repeats region is marked in (green). The ribbon schematics represent the α-helices (purple) and the short β-strands are illustrated by arrows (orange). The two highly branched glycosyl groups attached at asparagines residues 181 and 197 are shown (yellow). Figure source (http://www.fbs.leeds.ac.uk/staff/Hooper_N/prion.htm).

The structure of the globular domain of human PrP^C is homologous to various other mammals as it is conserved within many species (Lopez *et al.*, 2000; Lysek *et al.*, 2005). Notably, despite the low sequence homology between PrP^C in turtle, frog, or chicken and mammalian PrP^C, the major structural features of the protein are preserved in those non-mammalian species (Calzolai *et al.*, 2005). *PRNP* is abundantly expressed in CNS and cells of lymphoid tissues, but it is also expressed in other cell types (http://biogps.org/#goto=genereport&id=5621) and its predominant localisation is in cholesterol-rich lipid rafts or caveolae-like domains of the plasma membrane (Paratcha and Ibanez, 2002; Taylor and Hooper, 2006). Molecular dynamics simulations have suggested that the NH_2-terminal domain of PrP^C may interact with membrane lipids and traverse the surface of the membrane (DeMarco and Daggett, 2005).

1.2.3 PrP^C turnover in the cell

Notwithstanding the minute amount of truncated and transmembrane forms, the normal cellular form of prion protein is typically found a GPI-anchored to the outer leaflet of the cell membrane (Taylor and Hooper, 2006). Similar to other GPI-anchored proteins, PrP^C is synthesised as a precursor form with N- and C- terminal signal peptides and is translocated into the endoplasmic reticulum co-translationally. The N-terminal signal peptide is cleaved soon after translocation, and the C-terminal signal peptide is replaced by a preassembled GPI-anchor in a transamidation reaction. Subsequently, high-mannose glycans are added and one disulfide bond is formed. The glycans are then trimmed and modified as the protein traverses the Golgi complex on its way to the plasma membrane, where it is then anchored via its GPI moiety. PrP^C, like other GPI-anchored proteins, is predominantly found attached to low-density, detergent insoluble membrane domains (DRM), rich in cholesterol and sphingolipids (Naslavsky *et al.*, 1997; Taylor and Hooper, 2006)

Membrane rafts have been consensually described as small (10-200 nm), heterogeneous, highly dynamic, sphingolipid- and sterol-enriched domains that compartmentalise cellular processes and can occasionally be stabilised to form larger platforms through protein-lipid or protein-protein interactions (Pike, 2006). Despite some uncertainties, most of the evidence suggests a critical role for these distinctive membrane domains in the biology of the cell surface. Membrane rafts have been shown to be involved in pathogen invasion, regulation of protein and lipid sorting and also in intracellular signalling (Goot and Harder, 2001; Schuck and Simons, 2004; Pelkmans, 2005; Murphy *et al.*, 2006). Raft association of PrP^C was proposed to control the distribution of mature prion protein among distinct regions of the

plasma membrane, with sphingolipid or cholesterol depletion resulting in extensive redistribution of PrP^C at the cell surface (Galvan *et al.*, 2005).

The association of PrP^C with lipid rafts is dynamic, as a substantial fraction of the protein found in non-raft membrane, on its way to coated pits (Sunyach *et al.*, 2003). Study of cholesterol depletion also suggests that raft association is required for correct folding of PrP^C as well as for the export of the protein to the Golgi complex and correct glycosylation (Sarnataro *et al.*, 2004; Campana *et al.*, 2005). In addition, PrP^C that does not associate with rafts in the ER was shown to undergo conformational changes that modify protease sensitivity, suggesting that the immature protein may be misfolded and subjected to ER quality control mechanism (Sarnataro *et al.*, 2004). PrP^C subjected to endocytic recycling (Sunyach *et al.*, 2003) can be found in secretory cytoplasmic granules (Fournier *et al.*, 2000). Moreover, localisation of PrP^C in caveolae-like domains and evidence for PrP^C internalisation via non-clathrin coated vesicles has also been reported, leading to the contention that clathrin-coated vesicles may not participate in PrP^C trafficking (Vey *et al.*, 1996; Kaneko *et al.*, 1997a; Peters *et al.*, 2003; Marella and Chabry, 2004).

Caveolae are flask-shaped invaginations of the plasma membrane. The shape and structural organisation of caveolae (~ 60 nm in diameter) are conferred principally by caveolin, a dimeric protein that binds cholesterol. It inserts loop into the inner leaflet of the plasma membrane, and self-associates to form a striated caveolin coat on the surface of the membrane invaginations (Razani *et al.*, 2001). Although PrP^C lacks the expected signal sequences for intracellular internalisation, recent evidence supports a role of clathrin coated vesicles in the internalisation of PrP^C (Shyng *et al.*, 1994; Sunyach *et al.*, 2003; Taylor *et al.*, 2005; Taylor and Hooper, 2007). Additional

studies employing cell surface biotinylation, live cell microscopy of GFP-tagged PrP^C and electron microscopy also support the proposal that clathrin-coated vesicles and the classical endosomal organelles are involved in endocytosis of PrP^C (Shyng *et al.*, 1994; Magalhaes *et al.*, 2002; Sunyach *et al.*, 2003; Brown and Harris, 2003; Taylor *et al.*, 2005).

Moreover, the results from studies using dominant negative approaches support a role for dynamin and clathrin in the internalisation of PrP^C (Magalhaes *et al.*, 2002; Taylor *et al.*, 2005). Clathrin mediated endocytosis involves the concentration of high-affinity transmembrane receptors and their binding of ligands into "coated pits" on the plasma membrane (~ 120 nm in diameter), which are formed by the assembly of cytosolic coat proteins, mainly by clathrin. Molecules are carried into the cell by invagination and pinching-off of pieces of the plasma membrane in the form of endocytic vesicles encapsulated by polygonal clathrin coat (Conner and Schmid, 2003).

In addition to these findings, it has been proposed that lipoprotein receptor-related protein and/or laminin receptor may play a major role in the subcellular trafficking of PrP^C (Gauczynski *et al.*, 2001; Gauczynski *et al.*, 2006). However, it is still unknown whether the endocytic cycle of PrP^C is dependent on any physiological ligand. It may be presumed that cytosolic factors that participate in clathrin-mediated endocytosis, such as small GTP binding Rab proteins (Zerial and McBride, 2001) could have an important role in controlling PrP^C trafficking. Rab proteins, such as Rab 6, generally involved with retrograde transport of PrP^{Sc} to ER, may also regulate trafficking of PrP^C (Beranger *et al.*, 2002).

Evidence that PrP^C can be also found in the cytosol of various cultured cells and brain neurons have been shown (Ma and Lindquist, 1999; Ma and Lindquist, 2001; Yedidia *et al.*, 2001; Wang *et al.*, 2005; Dron *et al.*, 2009) and despite this, cytosolic PrP^C was originally proposed to be cytotoxic and perhaps involved in the neurodegenerative process found in prion diseases (Ma and Lindquist, 1999; Ma and Lindquist, 2001). However it has been found in the cytosol in subpopulations of neurons in several areas of normal healthy brain (Mironov *et al.*, 2003) and also cytosolic PrP^C was reported in various neuronal cell systems *in vitro* without exhibiting neurotoxic activity (Roucou *et al.*, 2003; Fioriti *et al.*, 2005). It is important to note that most studies pertaining to the pathogenic role of cytosolic PrP^C have been performed on genetically engineered cells in a non-infectious context (Dron *et al.*, 2009).

There are major unanswered questions concerning the physiological regulation of the endocytic cycle and of the fate of recycled PrP^C, following each cycle of internalisation. The misfolded, mutant or wild-type forms of PrP have been shown to be degraded by ER-associated degradation (ERAD) during which they are ubiquitinated and degraded by proteasome (Zanusso *et al.*, 1999; Jin *et al.*, 2000; Ma and Lindquist, 2001; Yedidia *et al.*, 2001; Ma *et al.*, 2002; Cohen and Taraboulos, 2003). A small fraction of endocytosed PrP^C was shown to be degraded by lysosomes (Shyng *et al.*, 1993; Mayer *et al.*, 1994), but a major fraction returns to the cell surface. In addition to the overall observations, studies originally intended to clarify the intracellular transfer of PrP^{Sc} have led to the observation that part of the recycled PrP^C is also secreted to the extracellular medium in association with exosomes (Fevrier *et al.*, 2004; Porto-Carreiro *et al.*, 2005; Robertson *et al.*, 2006). A

diagrammatic summary of PrP^C production, localisation and trafficking is illustrated in Figure 1.5.

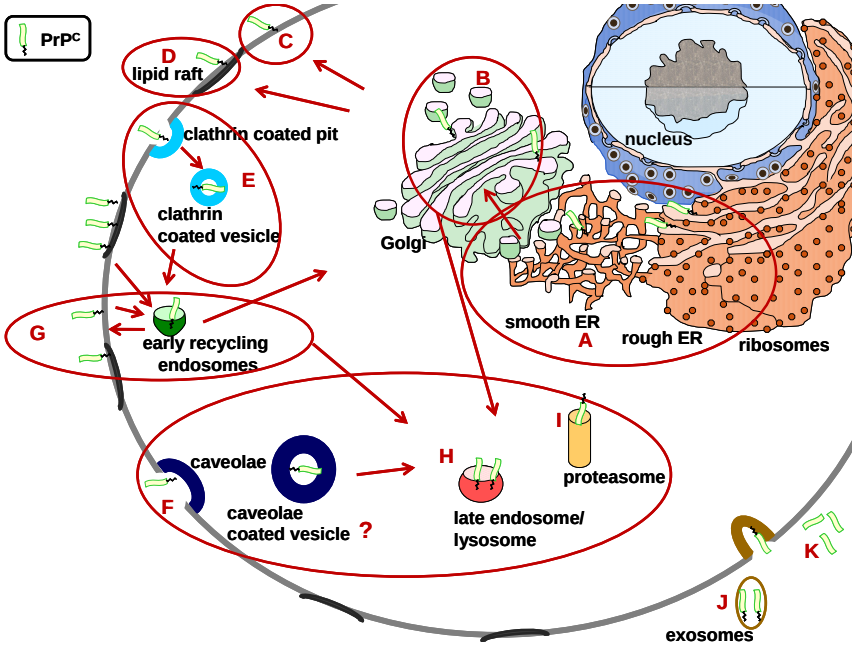


Figure 1.5: Cell surface localisation, routes of internalisation and trafficking of PrP^C
Precursor PrP^C is translocated co-translationally into endoplasmic reticulum (A) where the N-terminal signal sequence is cleaved by a signal peptidase. After translocation, the C-terminal GPI-signal peptide is replaced by a preassembled GPI anchor. Subsequently, the disulphide bond is formed and the glycans are added. The protein is then transported through the Golgi complex (B) to the cell surface where it resides in the plasma membrane (C) and in lipid rafts (D). PrP^C can be then endocytosed via clathrin-coated pits (E) or caveolae coated vesicles (F). PrP^C can be subsequently transported back to the cell surface via recycling vesicles (G). PrP^C can be degraded through the endosomal/lysosomal pathway (H), or by the proteasomes (I). Extracellular PrP^C can be associated both with exosomes (J) and without GPI-moiety (K) indicating phospholipase or protease mediated shedding.

1.2.4 Putative functions of PrP^C

The cellular prion protein has been the subject of intense research because it is considered to be the essential factor in the pathogenesis of prion diseases (Bueler *et al.*, 1993). Understanding the pathogenesis of TSEs accordingly requires a better definition of the functional properties of the normal prion protein. Despite marked PrP^C sequence conservation among species and advances in the determining PrP^C

structure, its constitutive function is still not precisely specified (Martins *et al.*, 2002).

1.2.4.1 PrP^C in CNS

PrP^C is especially abundant in brain regions characterised by a high degree of synaptic plasticity, for example the hippocampus (Sales *et al.*, 1998), and because of its predominant localisation at synapses (Manson *et al.*, 1992; Fournier *et al.*, 1995; Moser *et al.*, 1995; Sales *et al.*, 1998; Herms *et al.*, 1999; Moya *et al.*, 2000; Haeberle *et al.*, 2000; Laine *et al.*, 2001; Kovacs *et al.*, 2005; Godsave *et al.*, 2008) a role in synapse formation and function has been proposed for the normal cellular prion protein (Maglio *et al.*, 2004). Some combined data indicate that PrP^C could modulate neuronal excitability, synaptic activity, function and regulation (Collinge *et al.*, 1994; Herms *et al.*, 1999; Mallucci *et al.*, 2002), which are suggested to be the neural basis for some of the systemic brain functions attributed to PrP^C. Normal cellular prion protein was also indicated to be involved in the regulation of circadian rhythms and sleep patterns (Tobler *et al.*, 1996; Tobler *et al.*, 1997; Huber *et al.*, 1999; Huber *et al.*, 2002) and interestingly, links between sleep and memory/learning were previously observed (Born *et al.*, 2006). At least two definite molecular interactions of PrP^C with hippocampal cell surface proteins, LN and hop/STI1, may mediate effects of PrP^C on memory consolidation, and it was suggested that PrP^C modulates memory conservation through both these interactions. Further evidence for the hypothesis that PrP^C plays an important role in memory and cognition was observed in humans. The presence of valine at codon 129 of *PRNP* in at least one allele was linked with worsened cognitive performance in elderly individuals (Berr *et al.*, 1998; Kachiwala *et al.*, 2005).

Participation in transmembrane signalling processes involved in neuronal survival and neurite outgrowth mediated by PrP^C was also supported by several lines of evidence (Graner *et al.*, 2000; Chen *et al.*, 2003; Maglio *et al.*, 2004; Kanaani *et al.*, 2005; Lopes *et al.*, 2005; Santuccione *et al.*, 2005; Watts and Westaway, 2007; Aguzzi *et al.*, 2008a). A role in signal transduction was inferred from the localisation of PrP^C in lipid rafts and PrP^C has also been suggested to play role in neuroprotection by acting as a sensor of oxidative stress or by counterbalancing apoptotic signals (Kuwahara *et al.*, 1999; Milhavet *et al.*, 2000; Bounhar *et al.*, 2001; Roucou *et al.*, 2005; Roucou and LeBlanc, 2005; Khosravani *et al.*, 2008; Haigh *et al.*, 2009). The antiapoptotic function for PrP^C was proposed to occur via a PrP^C-mediated decrease in the expression of several proapoptotic proteins, as PrP^C expression in a PrP^C-null neuronal cell line can be shown to decrease the levels of p53, Bax, and caspase-3, and increased levels of Bcl-2 (Kim *et al.*, 2004). Several studies suggest a functional role of PrP^C in cellular copper metabolism and maintenance of the accurate oxidative balance, possibly through a regulation of intracellular copper transport (Brown *et al.*, 1997a; Brown *et al.*, 1997b; Pauly and Harris, 1998; Brown *et al.*, 1999; Brown *et al.*, 2001; bio Klamt *et al.*, 2001; Vassallo and Herms, 2003). A very recent study indicated that neuronal expression of PrP^C is essential for long-term peripheral myelin maintenance (Bremer *et al.*, 2010).

1.2.4.2 PrP^C in immune system

It has been proposed that the normal cellular prion protein plays an important role in the development and maintenance of the immune system, as well as in specific cellular immunological responses (Burwinkel *et al.*, 2004; Cartier *et al.*, 2005) as lymphocyte activation and proliferation (Li *et al.*, 2001; Bainbridge and Walker,

2005). The function of PrP^C outside the nervous and immune systems has not been clarified, but some putative roles have been suggested based on data obtained from transgenic mice and expression of PrP^C.

1.2.4.3 Other functions of PrP^C

Early embryonic and progressive expression of PrP^C mRNA and/or PrP^C protein is generally interpreted as evidence for developmental roles of PrP^C in cell proliferation, differentiation and programmed cell death (Lieberburg, 1987; McKinley *et al.*, 1987; Manson *et al.*, 1992; Sales *et al.*, 2002; Miele *et al.*, 2003). PrP^C may have an impact on the ability to regulate the self-renewal/differentiation status of stem cells (Miranda *et al.*, 2011) and sustain self-renewal of long-term hematopoietic stem cells under stressful conditions (Zhang *et al.*, 2006). PrP^C has also been suggested to stimulate cell signalling of a caveolin-1 dependent activation of the tyrosine kinase Fyn (Mouillet-Richard *et al.*, 2000). The suggestion of role in cell adhesion was based on observations that PrP^C interacts with the laminin receptor and its precursor (LRP/LR) as well as with the neuronal cell adhesion molecule (NCAM) (Rieger *et al.*, 1997; Rieger *et al.*, 1999; Graner *et al.*, 2000; Gauczynski *et al.*, 2001; Schmitt-Ulms *et al.*, 2001; Mange and Lehmann, 2002; Santuccione *et al.*, 2005; Parkyn *et al.*, 2008). Moreover, PrP^C has been proposed to be involved in the processing of sensory information by the olfactory system (Le Pichon *et al.*, 2009).

It seems unlikely that a protein as strongly conserved among species as PrP^C has evolved purely for the purpose of determining susceptibility to prion disease in organisms. If the function of PrP^C were entirely unrelated to prion disease pathogenesis, PrP^C would be just one of the many proteins whose function awaits clarification. Understanding of the functional roles of PrP^C has been confounded by

the striking lack of phenotype reported for the first *Prnp* knockout mouse (Bueler *et al.*, 1992). Moreover, *Prnp* ablation does not evoke disease, even when induced postnatally (Mallucci *et al.*, 2002). Therefore, prion pathology is unlikely to develop due to a simple loss of PrP^{C} function.

Nevertheless, it might seem unlikely that a single protein could have so many functions as described above. It needs to be taken in account that most of the suggestions regarding PrP^{C} function originate from single reports and there is a suspicion that each of these reports represents a specific aspect of a more complex system. It could be that the specific biological role of PrP^{C} is entirely dependent on the cell type in which it is expressed, the precise localisation within the cell in respect to the different plasma membrane environments and the level or differential expression of any processed variants of the wild-type protein or partner proteins. Additionally, studies of genetically modified animals are also controversial, perhaps dependent on the gene deletion approach used, the genetics and species background, and also compensatory mechanisms, all of which may influence the outcomes.

1.2.5 The abnormal disease associated form of the prion protein, PrP^{Sc}

1.2.5.1 The prion concept

The 'protein only' hypothesis postulates that prion replication results from a change in PrP conformation whereby an abnormal isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) is capable of inducing PrP^{C} to undergo a change of conformation into PrP^{Sc} without the presence of DNA (or RNA) (Griffith, 1967; Prusiner, 1982). PrP^{Sc} was shown to have the same amino acid sequence as the normal cellular prion protein, PrP^{C} (Bolton *et al.*, 1982;

Prusiner *et al.*, 1982; Chesebro *et al.*, 1985; Hope *et al.*, 1986; Stahl and Prusiner, 1991). The distinctive properties of either the infective or the cellular prion proteins are defined by post-translational modifications and the majority of the research in the prion field is focused on the mechanism by which PrP^{C} is converted into PrP^{Sc} (Pan *et al.*, 1993). Modification of the prion protein can result in a disease-associated protease-resistant isoform and as protease resistance does not specify that the isoform is capable of transmitting disease. The term PrP^{Sc} is generally used to denote the pathogenic, infectious isoforms and PrP^{res} the protease resistant isoform (the N-terminally truncated proteinase K resistant core form of PrP^{Sc} is called $PrP^{27–30}$ or PrP^{res}).

Arguments for prions being composed largely or exclusively of PrP^{Sc} include:

- No viral particles, bacteria, fungi or protozoan parasites have been found to be associated with prion diseases (Baker and Ridley, 1996b).
- Prions are encoded by a chromosomal gene of the host, not by a nucleic acid in the infectious scrapie prion particle (Alper *et al.*, 1966; Alper *et al.*, 1967; Oesch *et al.*, 1985; Basler *et al.*, 1986; Bellinger-Kawahara *et al.*, 1987; Bellinger-Kawahara *et al.*, 1988; Kellings *et al.*, 1992; Kellings *et al.*, 1993; Kellings *et al.*, 1994).
- PrP^{Sc} and scrapie infectivity copurify (Prusiner, 1982; Prusiner *et al.*, 1983; Hope *et al.*, 1986).
- Procedures that denature or hydrolyse PrP^{Sc} reduce the prion titres (McKinley *et al.*, 1983; Bolton *et al.*, 1984).
- PrP^{Sc} experimentally transmitted from one species to another results in PrP^{Sc} replication with amino acid sequence characteristic of the recipient sequence, suggesting that the hosts PrP^{C} was recruited to conversion into PrP^{Sc} by the donor agent (Scott and Fraser, 1989; Prusiner *et al.*, 1990; Weissmann, 1991).
- $PrP^{0/0}$ mice are resistant to prion infection, suggesting that PrP^{C} is essential for prion diseases (Bueler *et al.*, 1993; Prusiner *et al.*, 1993).
- The rate of PrP^{Sc} formation is rapidly increased in mice overexpressing PrP^{C} and leads to shorter incubation times (Scott *et al.*, 2000).
- Mutations in the human prion protein gene result in the formation of infectious PrP^{Sc} and are linked to inherited human prion diseases (Kovacs *et al.*, 2002).

- Synthetic prions produced in *Escherichia coli* can infect transgenic mice overexpressing truncated PrP^{C} (Legname *et al*., 2004).

Despite the increasing body of evidence in favour of the prion hypothesis, there is still ongoing debate about the exact composition of the infectious agent and the mechanisms by which pathogenesis occurs. Discovery of animal brain tissues containing high titres of prion infectivity but undetectable levels of PrP^{res} (Lasmezas *et al.*, 1997; Barron *et al.*, 2007) and the converse, the presence of high levels of accumulated PrP^{res} without apparent infectivity (Narang, 2002; Piccardo *et al.*, 2007) challenge the protein-only theory. More conventional explanations for TSE diseases based on virions, viruses, and other infectious agents containing small RNAs have been proposed (Rohwer, 1984; Bellinger-Kawahara *et al.*, 1988; Manuelidis *et al.*, 1988; Rohwer, 1991; Narang, 2002). The finding that retroviral RNA co-precipitates with PrP^{Sc} and that short (< 4 kb) RNA fragments are released after nuclease digestions from purified infectious fractions added weight to these proposals (Akowitz *et al.*, 1990; Akowitz *et al.*, 1994; Simoneau *et al.*, 2009). Moreover, addition of polyanions such RNA was observed to promote prion protein misfolding cyclic amplification, a cell free model of prion replication (Deleault *et al.*, 2007).

1.2.6 Proteolytic processing and characteristics of PrP^{C} and PrP^{Sc}

The synthesis of PrP^{C} is a rapid event (measured in minutes) and once in the plasma membrane this protein has a half-life of approximately 5 hours. In contrast, the formation of PrP^{Sc} has been estimated from minutes (Goold *et al.*, 2011) to hours and the proposed time for degradation exceeds 24 hours, however months have also been suggested (Caughey *et al.*, 1989a; Caughey *et al.*, 1989b; Borchelt *et al.*, 1990;

Peretz *et al.*, 2001). The two PrP isoforms, PrP^{C} and PrP^{Sc}, undergo different patterns of proteolytic cleavage which are highly dependent on their secondary and tertiary protein structure. The isoforms also have different glycosylation forms. Therefore PrP^{C} and PrP^{Sc} display different and complex banding patterns in Western blots (Lawson *et al.*, 2005).

Proteolytic processing of PrP^{C} termed α-cleavage is carried out by ADAM/TACE matrix metalloproteases and occurs at amino acid residue 111 yielding a 17 kDa fragment denoted C1 (Vincent *et al.*, 2001) which is further degraded in the cell. The N-terminal cleavage fragment of PrP^{C} is released extracellularly. The cleavage of PrP^{Sc} termed β-cleavage is carried out instead by calpains at amino acid residue 88 and this processing yields a 19-21 kDa fragment denoted C2. The C2 fragment corresponds to un-glycosylated $PrP^{27\text{-}30}$ (Chen *et al.*, 1995; Yadavalli *et al.*, 2004) and was found to accumulate both intracellularly and extracellularly (Caughey *et al.*, 1989a; Hope and Manson, 1991; Borchelt *et al.*, 1992) suggesting that the rate of PrP^{Sc} formation is most likely higher than its rate of degradation.

PrP^{C} structure contains 40% α-helix and only 3% β-sheet content (described in section 1.2.2), while PrP^{Sc} is composed of 30% α-helix and 43% β-sheet content (Pan *et al.*, 1993; Prusiner, 1998). It is proposed that PrP^{C} converts into its abnormal disease-associated isoform when the region corresponding to two α-helices at amino acid residues 108-144 fold into β-pleated sheets (Prusiner, 1998). These structural differences results in the two prion protein isoforms having different biochemical properties and characteristics (Table 1.3) (Figure 1.6).

Properties	Cellular form, PrP^{C}	Disease associated form, PrP^{Sc}
Isoform	Normal	Pathogenic
Location	Plasma membrane	Extracellular
α helices	40%	30%
β sheets	3%	43%
Solubility	Soluble under non-denaturing conditions	Insoluble in non-denaturing detergents
PK sensitivity	Protease sensitive	Partially protease resistant
Glycoforms	Mixture of un-, mono-, and di-glycosylated forms	Mixture of un-, mono-, and di-glycosylated forms
Turnover	Hours, rapidly metabolised ($T^1/_2$ ~ 5 hours)	Days, stable ($T^1/_2$ ~ 15 hours *in vitro*, ~ months)
Sedimentation properties	Monomeric	Forms aggregates

Table 1.3: Comparison of PrP^{C} and PrP^{Sc} properties

For many years prions were considered "non-degradable" and once formed almost impossible to break down entirely. This concept was based on the resistance of infectious prions to conventional procedures for sterilisation such as heat and formalin-treatment and a lack of convincing evidence for a cellular turnover of PrP^{Sc} in neuronal cells. However, recently described degradation of prions *in vivo* by immune cells, i.e. macrophages (Carp and Callahan, 1981; Beringue *et al.*, 2000), and *in vitro* by dendritic cells (Luhr *et al.*, 2002) and bovine macrophages (Sassa *et al.*, 2010) has generated great interest. The possibility that degradation of PrP^{Sc} might also occur in neurons was proposed in studies where constitutively prion-infected mouse neuroblastoma cell line treated with anti-PrP antibodies exhibited decreased levels of PrP^{Sc} in the cells (Peretz *et al.*, 2001; Enari *et al.*, 2001). This effect was thought to reflect an inhibition of the prion protein conversion by the antibody blocking the conversion process, paralleled by a cellular degradation of the pre-existing PrP^{Sc}. Studies of branched polyamine-induced clearance of PrP^{Sc} suggests that lysosomal proteases might be involved in the slow degradation of prions in infected cells (Supattapone *et al.*, 2001; Luhr *et al.*, 2004a; Luhr *et al.*, 2004b; Okemoto-Nakamura *et al.*, 2008), however the specific proteases responsible for this still remain to be determined. A recent observation hypothesizes that PrP^{Sc} inhibits

the 26S proteasome, thus precluding its degradation via that route (Kristiansen *et al.*, 2007; Goldberg, 2007; Deriziotis and Tabrizi, 2008). Stimulation of PrP^{Sc} degradation in prion infected cell lines has recently been described by using substances such as imanitib mesylate, trehalose and lithium that are able to increase the efficiency of autophago-lysosomal breakdown of proteins (Ertmer *et al.*, 2004; Aguib *et al.*, 2009; Heiseke *et al.*, 2009). However, none of these substances was effective in curing prion infected mice from disease (Yun *et al.*, 2007; Aguib *et al.*, 2009).

It has not been possible to produce a high resolution structure for PrP^{Sc}, because of its insolubility in the detergents used in the crystallisation approach. The highest resolution PrP^{Sc} structure thus far obtained was reported by Wille and colleagues (Wille *et al.*, 2002; Caughey *et al.*, 2009) and a trimetric arrangement of the PrP^{Sc} was suggested as a minimal assembly of the abnormal prion protein isoform (Govaerts *et al.*, 2004; DeMarco and Daggett, 2004; DeMarco *et al.*, 2006). PrP^{Sc} is partially resistant to proteinase K (PK) (McKinley *et al.*, 1983), while PrP^{C} is readily digested (Oesch *et al.*, 1985; Rubenstein *et al.*, 1986). Western blot of an SDS-PAGE of PrP^{C} and PrP^{Sc}, with and without PK digestion, is generally used not only in research, but also in diagnostic tests to distinguish between the two isoforms of the prion protein (Figure 1.6). In normal cell or tissue samples, three PrP^{C}-specific bands corresponding to a mixture on N-terminally truncated and differentially glycosylated forms of the prion protein are detected by Western blot. In prion infected cell or tissue samples, three PrP^{Sc}- specific bands are typically observed after PK digestion, corresponding to un- (~ 19-21 kDa), mono- (~ 24 kDa), and di- (~ 27-30 kDa) glycosylated forms.

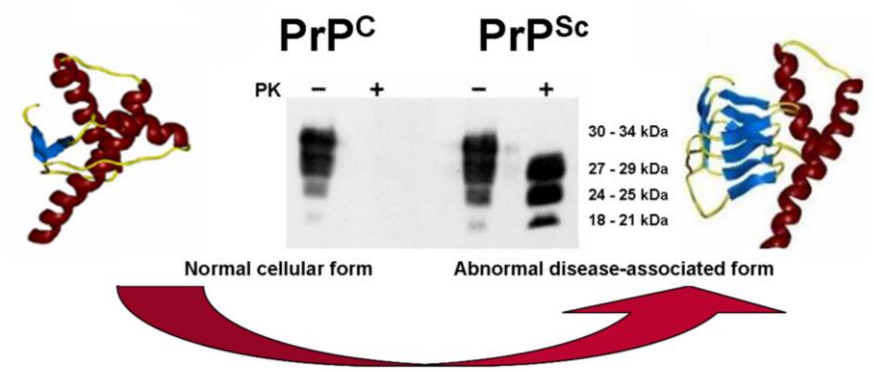


Figure 1.6: Fundamentally different structural features of prion protein isoforms (PrP^{C}, PrP^{Sc}) and their Western blot profiles with and without PK treatment
The cellular isoform (PrP^{C}) is present in the normal cell (left), and the pathogenic isoform (PrP^{Sc}) is a major component of the infectious material (right) (the α-helices are indicated in red; the antiparallel β-sheets are shown in blue). Western blot of a SDS gel electrophoresis of PrP^{C} and PrP^{Sc}, with and without PK digestion, show the characteristic PK sensitivity of PrP^{C} - disappearance after PK digestion. On the other hand, the three glycoforms of PrP^{Sc} (di-, mono-, and un-glycosylated) are still present, although shifted to lower M_r, representing the N-terminally truncated forms of PrP^{Sc}, called $PrP^{27–30}$ or PrP^{res}. It is noteworthy that PrP^{res} is fully infectious. Picture modified from (Riesner, 2002) and (http://www.cmpharm.ucsf.edu/cohen/).

1.2.7 Mechanism of prion protein conversion

The mechanism for the PrP^{C} to PrP^{Sc} conversion process has not yet been determined precisely. Two models have been proposed in order to explain the propagation of PrP^{Sc}, the "template-directed refolding" model (Gajdusek, 1988; Prusiner and DeArmond, 1990; Aguzzi and Polymenidou, 2004) and the "seeded nucleation" model (Come *et al.*, 1993; Telling *et al.*, 1994; Telling *et al.*, 1995; Kaneko *et al.*, 1997b; Aguzzi and Polymenidou, 2004). The latter is the more widely accepted and it proposes co-existence of the two isoforms of PrP in balance which strongly favours PrP^{C} in non-disease states. The monomeric form of PrP^{Sc} is thought to be harmless and it is the small oligomeric forms of PrP^{Sc} which propagate by recruiting PrP^{Sc} monomers to form PrP^{Sc} oligomers, which are proposed to be infectious. The "template-directed refolding" model proposes the spontaneous conversion as a rare

event which may be prevented by a high energy barrier between the two isoforms of PrP. The present PrP^{Sc} acts as a template for PrP^{C} to be recruited to form a pathogenic conformation (Aguzzi and Polymenidou, 2004; Caughey *et al.*, 2009). An alternative modified model supposes the existence of an auxiliary factor ("protein/cofactor X") (Telling *et al.*, 1994; Telling *et al.*, 1995; Kaneko *et al.*, 1997b) where PrP^{C} is in equilibrium with PrP* which binds to the putative protein X. PrP^{Sc} is able to interact with PrP*-protein X complex and trigger the conversion of PrP* to PrP^{Sc} (Telling *et al.*, 1995).

1.2.8 Possible site for conversion and PrP^{Sc} localisation in cells

Prion conversion is thought to occur predominantly at a site where two protein forms meet and physically interact (Caughey and Raymond, 1991; McKinley *et al.*, 1991; Arnold *et al.*, 1995; Prusiner, 1998; Barmada and Harris, 2005; Pimpinelli *et al.*, 2005; Godsave *et al.*, 2008). In contrast to PrP^{C}, PrP^{Sc} trafficking is less well studied and represents an important challenge, largely due to the lack of specific antibodies that can detect it *in situ* and the need to denature the protein by guanidine in order to expose PrP^{Sc} epitopes (Taraboulos *et al.*, 1990a).

A number of studies have implicated various intracellular compartments as the sites of prion conversion, by analyzing PrP^{C} and PrP^{Sc} subcellular distribution and trafficking in the brains of infected animals (Jeffrey *et al.*, 1994a; Jeffrey *et al.*, 1994b; Fournier *et al.*, 2000; Barmada and Harris, 2005; Godsave *et al.*, 2008), primary neurons (Shyng *et al.*, 1993; Sunyach *et al.*, 2003; Galvan *et al.*, 2005) and in constitutively infected cell cultures (Borchelt *et al.*, 1992; Beranger *et al.*, 2002). Knowledge of the precise site and mechanism of conversion is important, as

inhibition of conversion is a promising therapeutic option. The balance of evidence suggests that the conversion process occur either at the cell surface in association with lipid rafts, or more likely intracellularly along the endocytic pathway (Caughey and Raymond, 1991; Borchelt *et al.*, 1992; Jeffrey *et al.*, 1994b; Taraboulos *et al.*, 1995; Godsave *et al.*, 2008; Marijanovic *et al.*, 2009; Goold *et al.*, 2011).

Irrespective of its site of formation, PrP^{Sc} has been shown to localise to lipid rafts in the plasma membrane (Jeffrey *et al.*, 1994b; Vey *et al.*, 1996; Naslavsky *et al.*, 1997). A recent publication using a sophisticated cell culture model suggests that the plasma membrane is the primary site of prion conversion and that this occurs much faster than previously thought, within one minute after prion exposure (Goold *et al.*, 2011). In addition, PrP^{Sc} localisation in both early and late endosomes, as well as lysosomal compartments and multivesicular organelles has been observed (Caughey and Raymond, 1991; McKinley *et al.*, 1991; Borchelt *et al.*, 1992; Laszlo *et al.*, 1992; Jeffrey *et al.*, 1994a; Arnold *et al.*, 1995; Fournier *et al.*, 2000; Pimpinelli *et al.*, 2005; Godsave *et al.*, 2008; Caughey *et al.*, 2009). Accumulation of PrP^{Sc} was also observed in the perinuclear Golgi region (Barmada and Harris, 2005). A more recent report described accumulation of high amounts of PrP^{Sc} in the endosomal recycling compartments (Marijanovic *et al.*, 2009). Under conditions of mild proteasome inhibition, cytoplasmic PrP aggregates (e.g. aggresomes) were also observed (Ma *et al.*, 2002; Kristiansen *et al.*, 2005). The presence of extracellular PrP^{Sc} was reported to be associated with exosomes (Fevrier *et al.*, 2004; Robertson *et al.*, 2006; Vella *et al.*, 2007; Vella *et al.*, 2008a; Vella *et al.*, 2008b), thereby suggesting a possible process of cell-to-cell propagation of infection (Figure 1.7).

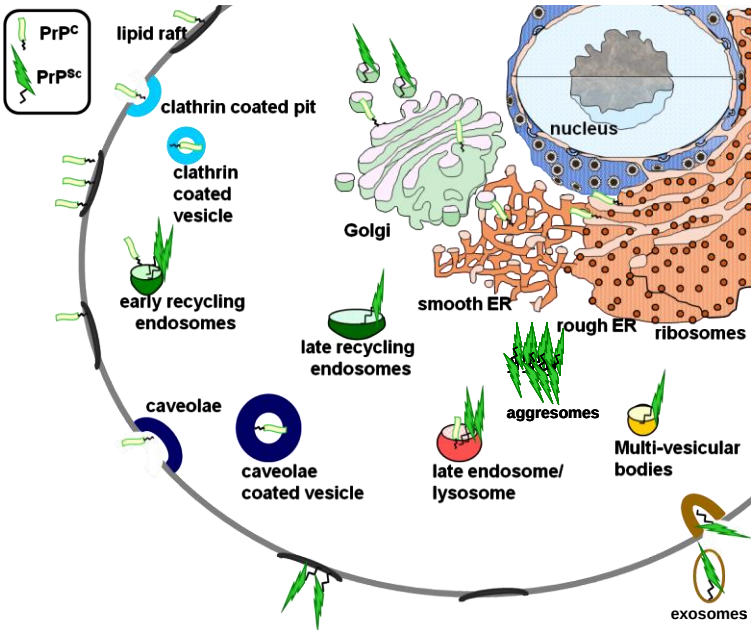


Figure 1.7: Proposed sites of PrP^{Sc} localisation in constitutively infected cells
Prion infected cell culture models established to date revealed PrP^{Sc} presence and accumulation at the cell surface in association with lipid rafts, early endosomes, trans-Golgi compartments, late endosomes, lysosomes, multi-vesicular bodies, aggresomes and exosomes. Some of these locations were also proposed as a likely site of PrP^{C} to PrP^{Sc} conversion.

Another recent investigation has proposed the mediation of PrP^{Sc} *in vitro* intercellular transfer between neuronal cells and between bone-marrow derived dendritic cells and neurons by means of tunnelling nanotubes (TNTs) (Gousset *et al.*, 2009). Since dendritic cells can interact with peripheral neurons in lymphoid organs (Huang *et al.*, 2002), TNT-mediated intercellular transfer (Gousset *et al.*, 2009) was proposed to spread PrP^{Sc} from the peripheral site of entry to the PNS by neuroimmune interactions with dendritic cells and to be involved in the spreading of PrP^{Sc} within neurons in the CNS *in vivo* (Aucouturier *et al.*, 2001; Gousset *et al.*, 2009).

Although the data do seem to be somewhat contradictory this may be caused by the different approaches used (whole animals vs. cultured cells), cell types (neurones,

neuroblastoma cells etc.) and experimental paradigms (chronic infection vs. acute exposure) in these studies.

1.2.9 Neurotoxicity

In neurons, PrP^{Sc} has been proposed to localise to both chemical (Kitamoto *et al.*, 1992; Fournier *et al.*, 2000; Kovacs *et al.*, 2005) and electrical (Kovacs *et al.*, 2005) synapses. However, a recent cryo-EM study did not show any evidence of PrP^{Sc} synaptic localisation (Godsave *et al.*, 2008). Neuronal damage has been suggested to result from a loss of the normal prion protein function or a gain of toxic properties of disease-associated prion protein isoform (Chiesa and Harris, 2001).

The potential role of PrP^{C} loss of function in prion diseases has been predominantly studied in PrP^{C} knock-out mice. These are described as normal in their development and behaviour (Bueler *et al.*, 1992; Manson *et al.*, 1994). However, some signs of slight alterations in neuronal function, such as disruption in synaptic function in PrP^{C}-deficient mice have been reported (Collinge *et al.*, 1996; Tobler *et al.*, 1996; Colling *et al.*, 1996; Herms *et al.*, 2001; Carleton *et al.*, 2001; Fuhrmann *et al.*, 2006), but no synaptic loss, myoclonic seizures or neuronal degeneration could be observed. Neurotoxic properties of PrP^{Sc} have been proposed based on observations showing that PrP^{Sc} colocalises with the histopathological changes observed in prion diseases (DeArmond *et al.*, 1987; Jendroska *et al.*, 1991; Jeffrey *et al.*, 1994a). Moreover, synthetic fragments of prion protein displayed neurotoxic properties and a tendency to aggregate into fibrils *in vitro* (Forloni *et al.*, 1993).

As previously mentioned, several inherited prion diseases contain only low levels of PrP^{res} in the brain of affected individual despite a fatal clinical disease (Tateishi *et al.*,

1992; Medori *et al.*, 1992a; Medori *et al.*, 1992b; Collinge *et al.*, 1995). Further arguments against extraneuronal PrP^{Sc} neurotoxicity have been proposed in a study by Brandner and colleagues, where they transplanted neuronal tissue from PrP^{C} overexpressing mice to PrP^{C} knock-out mice that were subsequently inoculated with infectious prions. High levels of PrP^{Sc} accumulation and prion disease characteristic histopathological changes were observed in the transplanted grafts. However, these signs were completely absent in PrP^{C} deficient tissue despite the graft-derived PrP^{Sc} being spread extracellularly to the host brain tissue (Brandner *et al.*, 1996a). Study of prion disease inoculated mice expressing mutant PrP^{C} lacking the GPI-anchor developed minimal brain pathology and neurological dysfunction despite the accumulation of multiple PrP^{Sc}-containing amyloid plaques (Chesebro *et al.*, 1985). Moreover, it was shown that depletion of neuronal PrP^{C} in mice with established CNS scrapie infection prevented progression to clinical stage of prion disease and resulted in long-term survival of infected animals (Mallucci *et al.*, 2003). Additionally, no cytopathic effect of PrP^{Sc} can be observed in prion infected cells cultured *in vitro* (Naslavsky *et al.*, 1997). Overall these studies indicate that neither loss of PrP^{C} function nor gain of toxic PrP^{Sc} properties can fully explain prion-induced neurodegeneration. One explanation, therefore is that the $PrP^{C} \rightarrow PrP^{Sc}$ conversion process, which might include a toxic intermediate PrP-species (Hill *et al.*, 2000; Hill *et al.*, 2003; Collinge and Clarke, 2007) may trigger signalling that could alter cell functions and/or initiate some toxic cascades contributing to neurodegeneration processes in prion diseases (Sandberg *et al.*, 2011).

1.3 PRION DISEASES

1.3.1 Human prion disease

1.3.1.1 Molecular classification of PrP^{Sc}

Prion diseases are rare and fatal neurodegenerative diseases. At microscopic level, they exhibit four principal neuropathological features: spongiform vacuolation, extensive neuronal loss, astrocytosis and microglial proliferation, as well as an accumulation of the disease-associated isoform of the prion protein in the brain. Among all TSEs the human diseases are unique as they can be sporadic, inherited, or acquired in their origin (Wadsworth *et al.*, 2003; Gambetti *et al.*, 2003; Ironside and Head, 2004). The human prion diseases primarily affect the nervous system; however, vCJD has been shown to have a peripheral involvement in secondary lymphoid tissues (Kong and Bessen, 2008).

The causative factor for all prion diseases is thought to be the misfolding of the host PrP^{C} protein into the disease-associated prion protein conformation, PrP^{Sc}. Exposure of naïve host to exogenous PrP^{Sc} can lead to prion protein misfolding and result in the onset of neurodegenerative disease. Hence, prion diseases are transmissible, but are caused by a novel unique pathogen that lacks a prion-specific nucleic acid genome (Prusiner, 1982; Prusiner, 1998). Nevertheless, the cause of sporadic CJD, the commonest form of human prion disease, remains unknown.

Biochemical analysis employing PK digestion followed by Western blot analysis reveals PrP^{Sc} heterogeneity in the form of the size of the protease-resistant core fragment (PrP^{res}) and in the relative amounts of the three possible glycoforms of the protease-resistant core fragments. Proteolysis under defined conditions digests approximately 7 kDa of the N-terminus leaving a protease-resistant core fragment of PrP^{Sc} intact. Different PrP^{Sc} types differ in the extent of N-terminal (and sometimes

C-terminal) truncation following proteolysis with PK. Generally, there are three protease-resistant polypeptide fragments present differing in their molecular weight due to the occupancy of the two N-linked glycosyl groups and these are designating glycoforms of the prion protein. Using a combination of PrP^{res} fragment size and glycoform ratio has proven to be very useful for the classification of different human prion disease phenotypes according to molecular and genetic criteria (Monari *et al.*, 1994; Collinge *et al.*, 1996; Parchi *et al.*, 1996; Parchi *et al.*, 1997; Hill *et al.*, 2006). According to Parchi and Gambetti nomenclature, PrP^{Sc} can be classified into three operational groups denoted type 1, type 2 (A or B) (Figure 1.8), and ~ 8 kDa low molecular weight fragment type (Parchi *et al.*, 1996; Parchi *et al.*, 1997). The A or B types are referred to the glycosylation site occupancy. Type A is characterised by a prominent mono-glycosylated (or rarely un-glycosylated) PrP^{Sc} fragment and the type B by high occupancy of both potential glycosylation sites (Parchi *et al.*, 1997; Head *et al.*, 2004b). Recently, a smaller C-terminal fragments consisting of 12-13 kDa were identified in some forms of sCJD (Zou *et al.*, 2003; Notari *et al.*, 2008) but these could only be detected when antibodies recognising the extreme C-terminus of PrP were used.

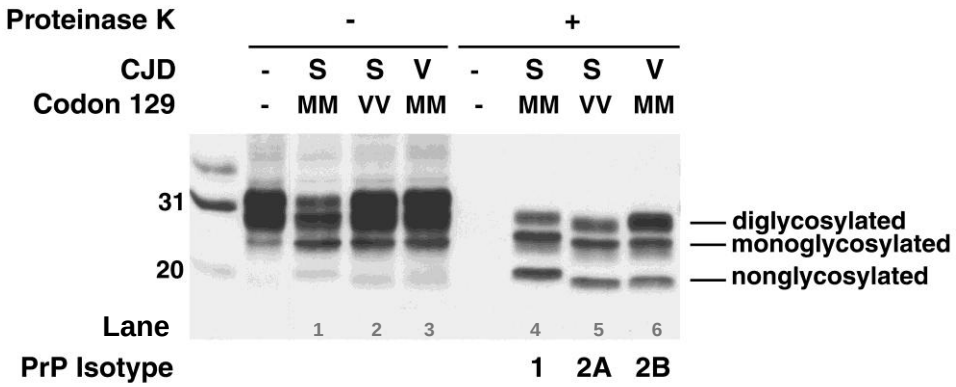


Figure 1.8: Representation of PrP^{Sc} types found in human prion diseases
Western blot analysis of sporadic CJD (S) or variant CJD (V) brain sample shows the three major protein polypeptide glycoforms (lane 1-3). Samples were analysed without (-) and with (+) proteinase K digestion. The limited digestion of PrP^{Sc} with PK, which removes the N-terminal proportion of the prion protein, reveals PrP^{res} types (lanes 4-6). The migration of the protease-resistant PrP^{res} glycoforms is marked (right). The shading intensity is indicating the relative amount of each PrP polypeptide in relation to the other glycoforms. The molecular weight is indicated in kilodaltons (left). PrP^{res} isotypes 1, 2A and 2B are defined by the molecular weight of the un-glycosylated PrP^{res} polypeptide and by the ratio of the three PrP^{res} glycoforms (marked beneath). Figure was a kind gift from Dr Head.

The majority of sCJD cases were found to be PrP^{Sc} type 1. This is also true for familial CJD cases (Gambetti *et al.*, 2003) and a small amount of type 1 PrP^{Sc} has been suggested to be present in all CJD cases (Yull *et al.*, 2006; Parchi *et al.*, 2009). Type 2A PrP^{Sc} is found in a minority of sCJD, sporadic fatal insomnia (FFI) and some familial CJD (fCJD) cases. Many sCJD patients (20% or more) have both type 1 and type 2 present in the same or different brain regions (Puoti *et al.*, 1999; Head *et al.*, 2004a; Head *et al.*, 2004b; Schoch *et al.*, 2006; Uro-Coste *et al.*, 2008). In vCJD, the PrP^{Sc} glycoforms have a similar molecular weight to those found in type 2A PrP^{Sc}, but the ratio of the three PrP^{Sc} glycoforms differs (predominance of di-glycosylated form) and thus vCJD "glycoform signature" is designated as type 2B PrP^{Sc} (Figure 1.8). In Gerstmann-Sträussler-Schienker syndrome a 7-8 kDa PrP^{Sc} polypeptide was recognised associated with various pathogenic mutations sometimes alone and in other cases or regions in combination with type 1 PrP^{Sc} (Parchi *et al.*,

1998; Piccardo *et al.*, 1998; Piccardo *et al.*, 2001). The majority of UK iCJD cases showed to be of the Parchi and Gambetti type 2A (in association with *PRNP* codon 129 MV or VV) (Heath *et al.*, 2006) reviewed by (Ironside, 2008).

1.3.2 Idiopathic forms of human prion diseases

Sporadic forms of human prion diseases comprise two prion disease phenotypes: sporadic Creutzfeldt-Jakob disease (sCJD) and sporadic fatal insomnia (sFI). In sFI the PrP^{Sc} level is much lower when compared to sCJD cases and immunostaining for PrP is usually negative or minimal in limited brain areas (Parchi *et al.*, 1999a; Gambetti *et al.*, 2003). Moreover a novel phenotype of human sporadic prion disease with distinct clinical and neuropathological features was recently identified by Gambetti and co-workers and termed protease-sensitive prionopathy (PSPr) (Gambetti *et al.*, 2008), which was later changed to variably protease sensitive prionopathy (VPSPr) (Zou *et al.*, 2010). Abnormal PrP associated with this phenotype was shown to be less aggregated and largely sensitive to PK digestion. Proteolytic treatment produces a "ladder-like" profile of PrP^{res} after electrophoretic migration ranging from ~ 6 to 29 kDa (Gambetti *et al.*, 2008). Recently, some additional cases of VPSPr were identified in the UK and the Netherlands (Head and Ironside, 2009; Jansen *et al.*, 2010; Head *et al.*, 2010).

1.3.3 Hereditary forms of human prion diseases

Inherited prion diseases are associated with autosomal-dominant pathogenic or insertional *PRNP* gene mutations (shown in the upper half of Figure 1.3), many of which exhibit high penetrance in the population and represent 10-15% cases of

human prion diseases (Masters *et al.*, 1981; Windl *et al.*, 1996; Windl *et al.*, 1999; Gambetti *et al.*, 2003; Kovacs *et al.*, 2005). Autosomal dominant inheritance of CJD was first reported almost 90 years ago (Kirschbaum, 1924; Meggendorfer, 1930). However, most *PRNP* mutations are heterozygous and not all appear to be pathogenic (Chen *et al.*, 1997; Silvestrini *et al.*, 1997). Variability in the clinical and pathological findings such as age of onset or duration was observed to be linked with particular mutation (Prusiner and Scott, 1997; Kovacs *et al.*, 2002; Kovacs *et al.*, 2005). It was also proposed that some carriers of some mutations in *PRNP* (low penetrance) can live long healthy lives without developing clinical signs of prion disease (Pocchiari *et al.*, 1993; Spudich *et al.*, 1995; Mitrova and Belay, 2002). The pathogenic mutations identified up to date (over 30 pathogenic mutations) are classified as point mutations resulting in amino-acid substitution or premature stop codon, and insertion of additional octapeptide repeats (Mead *et al.*, 2006). These mutations are thought to cause changes in the amino acid sequence, thus impairing the stability of the tertiary structure of the prion protein which is then more readily convertible to the disease associated form (Wadsworth *et al.*, 2003). According to clinicopathological features of the disease phenotype the inherited prion diseases are classified as familial CJD (fCJD), Gerstmann-Sträussler-Schienker disease (GSS) and fatal familial insomnia (FFI).

1.3.4 Acquired form of human prion diseases

The acquired prion diseases account for less than 1% of all human prion disease cases and these include kuru, iatrogenic CJD (iCJD) and variant CJD (vCJD) and the prion infection is either associated with accidental transmission via medical or

surgical practices or acquired orally. The acquired human prion diseases caused by human-to-human transmission of the infectious agent are iCJD, kuru, and the bovine-to-human caused prion disease is vCJD.

Kuru, first reported in 1959 (Gajdusek and Zigas, 1957; Gajdusek and Zigas, 1959), was linked to ritualistic endocannibalism the of indigenous tribes in the Eastern Highlands province of Papua New Guinea, during which bodies of deceased relatives were consumed as a mark of respect (Gajdusek, 1977; Alpers, 1979). The disease has gradually declined with cessation of cannibalism in the late 1950s and kuru is now thought to be extinct as no children born after cannibalism ceased have developed it.

1.3.4.1 Variant CJD

vCJD was first described in 1996 (Will *et al.*, 1996) as a new form of CJD. Histopathological, biochemical, and epidemiological evidence all suggests that vCJD is most likely to be a result of cross-species transmission by orally ingested bovine spongiform encephalopathy (BSE) prion agent via BSE-contaminated food products (Will *et al.*, 1996; Collinge *et al.*, 1996; Lasmezas *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997a; Prusiner, 1998; Scott *et al.*, 1999). It is now widely accepted that vCJD and BSE are caused by the same prion strain (Hill *et al.*, 1997a; Ward *et al.*, 2006) and vCJD is so far the only known example of zoonotic human prion disease. Removal of the meat and bone meal supplements from livestock feed in the late 1980s has significantly reduced the prevalence of BSE in UK. As a result, human exposure to BSE has also been greatly reduced consequently resulting in a reduced vCJD incidence in human population, as shown on the Figure 1.9.

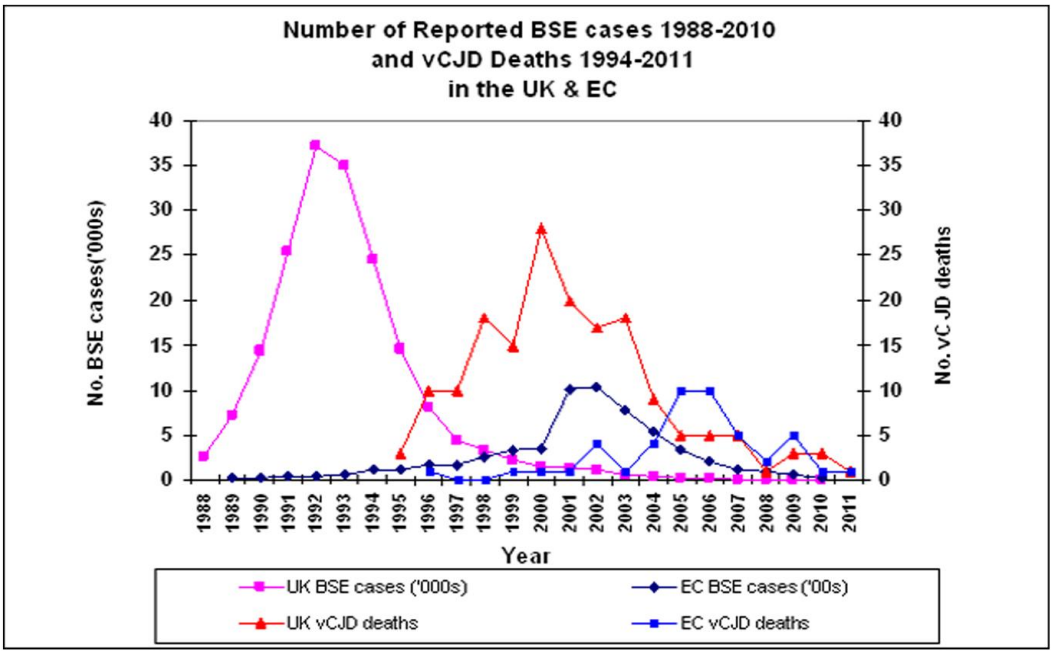


Figure 1.9: Epidemiology of BSE and vCJD in UK and Europe
The survey of BSE cases (indicated pink and dark blue) is represented by a number of cases in thousands and vCJD (indicated red and light blue) is represented by a number of cases in hundreds. Graph is showing the course development of BSE and vCJD in United Kingdom (UK) (pink, red) and European countries (EC) (dark and light blue) from 1988 to 2011. The BSE figures are only until year 2010 as the UK BSE data were only given to 2010 and in Europe there has only been one case in the Netherlands as at January 2011 and 2 in Switzerland as at May 2011. Figure kindly provided by Jan MacKenzie (NCJDRSU Study Co-ordinator).

The clinicopathological features of vCJD are distinct from most of the human prion diseases (Ironside, 2002; Ironside and Head, 2004). Unlike sporadic and inherited CJD, vCJD affects also the lymphoreticular system (Hill *et al.*, 1997a; Hill *et al.*, 1999; Wadsworth *et al.*, 2001), most likely as a reflection of the peripheral route of exposure (Ramasamy *et al.*, 2003). vCJD is typically found in younger individuals (with mean age of onset around 28 years, ranging between 12 to 74 years of age) when compared to sCJD (with mean of 65 years at onset). The clinical duration of the illness in vCJD affected individuals is longer (median time 13 months) than the 4 months for sCJD (MM1), but comparable to that of 16 months for sCJD (MM2) individuals.

As of September 2012, 176 primary cases of vCJD have been identified in the UK (176 dead, 0 alive) and 51 from outside UK (49 dead, 2 alive) (http://www.cjd.ed.ac.uk). All symptomatic sufferers of vCJD have been identified in UK and France to be homozygous for methionine (M) at *PRNP* codon 129 genotype (Brandel *et al.*, 2009; Bishop *et al.*, 2009; Peden *et al.*, 2010). However, individuals who are heterozygous or homozygous for valine (V) at codon 129 appears to be susceptible to vCJD/BSE prion agent, although may be found to have a more prolonged incubation period when compared to MM individuals as suggested by the appendix study (Hilton *et al.*, 2004; Ironside *et al.*, 2006; Kaski *et al.*, 2011) and recent transmission studies employing gene-targeted human transgenic mice (Bishop *et al.*, 2006). The first case recognised to be transmitted horizontally via blood transfusion from pre-sypmtomatic vCJD sufferer (MM at codon 129) was in 2003. This case was identified through a collaborative study between UK National Blood Services, the National CJD Research and Surveillance Unit, and the Office of National Statistics (Llewelyn *et al.*, 2004; Hewitt *et al.*, 2006).

Further cases of human-to-human transmission of vCJD linked to blood transfusion and blood product therapy were subsequently identified and these were two individuals of MM genotype (Wroe *et al.*, 2006; HPA, 2007) and two of MV genotype (Peden *et al.*, 2004; Peden *et al.*, 2010). The biological strain properties of PrP^{res} originating from vCJD (or BSE) were shown to remain unaltered after secondary human-to-human transmission (Bishop *et al.*, 2008). Subsequently, neuropathological features and PrP^{res} profiles of the secondary cases were found to be indistinguishable when compared to those of primary cases (Wroe *et al.*, 2006). However, it can be speculated that there must be a variety of environmental,

individual, genetic, or cellular determinants which may influence susceptibility or resistance to BSE/vCJD because the estimated number of individuals exposed, and predictive models of how many individuals may acquire the disease (Ghani *et al.*, 2000; Valleron *et al.*, 2001; Ferguson *et al.*, 2002; Ghani *et al.*, 2003; Huillard d'Aignaux *et al.*, 2003) were dramatically greater than the actual numbers of patients who have as yet developed vCJD.

The remarkably long incubation period that proceeds the neurological symptoms indicate that important events in prion disease pathogenesis might take place in extranervous sites (Aucouturier and Carnaud, 2002). Following peripheral infection, PrP^{Sc} can be detected in the spleen, lymph nodes and other lymphoid tissues long before CNS involvement (Eklund *et al.*, 1967; Kimberlin and Walker, 1979; Hilton *et al.*, 1998). However, the spleen might not be the principal organ involved in infection via the oral route as infectivity can be found first in the Peyer's patches (Kimberlin and Walker, 1979). This indicate that the gut-associated lymphoid tissues (GALT) could have a similar processing and replicative function for inocula routed intestinally (van Keulen *et al.*, 1996; Heggebo *et al.*, 2000; Andreoletti *et al.*, 2000; Heggebo *et al.*, 2002). Interestingly, splenic PrP^{Sc} accumulation is not detectable in cattle naturally infected with BSE (Somerville *et al.*, 1997), although it can be found in experimental animal models including BSE-infected mice (Maignien *et al.*, 1999) and sheep (Foster *et al.*, 2001).

The peripheral lymphoid organs are believed to be important and perhaps indispensable in the transmission process and development of the neurodegenerative (Aucouturier and Carnaud, 2002). The cell types likely to be involved in propagations of prions were suggested to be low density (Clarke and Kimberlin,

1984), long-lived and mitotically quiescent cells (Fraser and Farquhar, 1987). Follicular dendritic cells (FDCs) are believed to play a key role in the peripheral pathogenesis of prion diseases and have long been suggested to be a major site of prion replication and accumulation in the germinal centres of spleen, lymph nodes, and mucosa-associated lymphoid tissue following experimental or natural contamination with infectious prions (Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Hill *et al.*, 1997a; Hill *et al.*, 1997b; Mabbott *et al.*, 1998; Sigurdson *et al.*, 1999; Andreoletti *et al.*, 2000; Beekes and McBride, 2000; Mabbott *et al.*, 2000; Heggebo *et al.*, 2002; Ramasamy *et al.*, 2003; reviewed by Mabbott and MacPherson, 2006). FDCs of the germinal centres of lymphoid organs exhibit all these characteristics and moreover they are characterised by high levels of PrP^{C} expression (McBride *et al.*, 1992). Functional FDCs appear to be essential (Mabbott *et al.*, 2000; Montrasio *et al.*, 2001; reviewed by Mabbott and MacPherson, 2006) for the obligate peripheral phase of infection in some murine scrapie models, but it is still not entirely clear whether they are the only site of lymphoreticular prion replication or accumulation (Blattler *et al.*, 1997; Kaeser *et al.*, 2001).

1.3.4.2 Iatrogenic CJD

iCJD is the second most common acquired human prion disease after kuru. These cases are a result of accidental transmission of prion infection during the course of medical treatments or surgical procedures, reviewed (Collins *et al.*, 2004; Aguzzi *et al.*, 2008b). The first reported case of iCJD was in 1974 and was associated with transplantation of a corneal graft derived from a donor who had died of sCJD (Duffy *et al.*, 1974). iCJD has been identified in over 400 cases world-wide over the past 30 years (Duffy *et al.*, 1974; Will, 2003; Brown *et al.*, 2006) including 65 cases from

UK (http://www.cjd.ed.ac.uk). The majority of cases were acquired by transmission of prions via contaminated human dura mater grafts, and human pituitary hormones derived from pooled tissues from deceased individuals that included material from an individual or individuals suffering from unrecognised TSEs. A minority of cases were associated with neurosurgery using contaminated EEG electrode implantations, surgical operations using contaminated instruments or apparatuses, and transplantation of corneal graft from undiagnosed CJD infected patients (Bernoulli *et al.*, 1977; Masters and Richardson, Jr., 1978; Davanipour *et al.*, 1984; Powell-Jackson *et al.*, 1985; Brown *et al.*, 1985a; Brown *et al.*, 1985b; Buchanan *et al.*, 1991; Fradkin *et al.*, 1991; Healy and Evans, 1993; Brown *et al.*, 2000; Collins *et al.*, 2004; Brown *et al.*, 2006). The incubation period can range from 2 to 38 years (median period 7 years) (Heckmann *et al.*, 1997).

Molecular genetic studies have shown that the majority patients developing iCJD after receiving pituitary-derived human growth hormone therapy were homozygous for either methionine or valine at codon 129 of the *PRNP* gene (Collinge *et al.*, 1991a; Brown *et al.*, 1994b). The study of UK iCJD cases revealed notably higher number of cases of VV genotype indicating possible contamination by infectious material of VV genotype (sCJD) (Brown *et al.*, 1992).

1.3.5 Animal prion diseases

Prion diseases have also been found to afflict a number of different animal species, either occurring naturally, or caused by consumption of prion contaminated food or induced experimentally as a tool for study of prion diseases.

Scrapie, naturally occurring in sheep and goats, was the earliest described prion disorder. The term scrapie was coined from observations of behavioural changes in afflicted animals which were "scraping" or rubbing themselves against surfaces in the field. Scrapie is found world-wide, except in Australia and New Zealand and has never been shown to pose a threat to human health (Brown, 1998).

Chronic wasting disease (CWD) is another naturally occurring prion disease which afflicts non-domesticated or captive wildlife deer, elk, and moose (Williams and Young, 1982; Williams and Young, 1993; Williams, 2005). CWD is found mainly in North America and Canada (Sigurdson, 2008). As yet, no evidence has been shown for CWD transmission to humans.

A number of prion diseases afflicting other animal species have been recognised to be associated with BSE through consumption of prion contaminated meat and bone meal feed. Spongiform encephalopathy was found in domestic cats, feline (FSE) and mink (TME), exotic ruminants (EUE) and primates kept in Zoos (Leggett *et al.*, 1990; Wyatt *et al.*, 1991; Kirkwood and Cunningham, 1994; Prusiner, 1998; Bons *et al.*, 1999). In each case, association with BSE was geographically evident and was verified by experimental transmission studies (Fraser and Foster, 1994; Collinge *et al.*, 1996; Bruce *et al.*, 1997; Fraser, 1998; Baron and Calavas, 2005; Barron *et al.*, 2007).

BSE was first identified in the late 1980s in UK as an epizootic epidemic of previously unknown disease of cattle (Wells *et al.*, 1987; Wilesmith, 1988; Anderson *et al.*, 1996) and it was classified as prion disease upon the discovery of protease-resistant prion protein in the brain material of the affected cattle (Hope *et al.*, 1988). BSE was a result of industrial cannibalism in which cattle feed was enriched with

meat and bone meal derived from unrecognised prion-infected animal sources (rendered sheep or cattle offal) (Wilesmith, 1991; Wilesmith *et al.*, 1992). The meat and bone meal animal feed was also exported to many other countries, resulting in BSE identified throughout Europe and in some North American and Asian countries (Smith and Bradley, 2003). Since 1988 this practise has been forbidden in the UK. The BSE epidemic reached its peak in 1992 with over 35,000 cattle infected. By 2003 BSE had become a rare disease, but still has not been completely eradicated from British or European cattle (http://vla.defra.gov.uk/science/docs/sci_tse_stats _gen.pdf).

No evidence of PrP^{Sc} accumulation in lymphoid tissues or in other systematic tissues in field cases of BSE was shown (Wells *et al.*, 2005). BSE was proven as transmissible to mice, cattle, sheep, pigs and non-human primate marmoset, domestic cats, wild cats and range of exotic ungulates in zoos (Dawson *et al.*, 1990a; Dawson *et al.*, 1990b; Baker *et al.*, 1993a; Baker *et al.*, 1993b; Kirkwood and Cunningham, 1994; Fraser and Foster, 1994; Baker and Ridley, 1996a; Bruce *et al.*, 1997). The BSE agent was shown to retain its biological identity after crossing between species either naturally or experimentally (Bruce *et al.*, 1994; Bruce *et al.*, 1997; Hope *et al.*, 1999; Stack *et al.*, 2002).

Extensive surveillance for BSE in Europe and Japan has identified several cases of "atypical" BSE which has been termed bovine amyloidotic spongiform encephalopathy (BASE) characteristic with distinctive neuropathological profile from that found in conventional BSE comprising relative lack of neuronal vacuolation and PrP-immunoreactive amyloid mini-plaques in the brain stem (Biacabe *et al.*, 2004; Casalone *et al.*, 2004).

1.4 MODELS FOR STUDYING PRION DISEASES

1.4.1 In vivo models for studying prion diseases - Animal models

In medical research, animal diseases are often used as convenient and relevant models of human disease, and that is the case for human prion diseases. Transmission studies in the field of prion research have been undertaken for many years, more commonly using scrapie, as this was the first prion disease to be characterised. Accordingly, sheep and goats were originally used to for such studies and sheep are still used today. However, the use of small rodents (mice, hamsters and others) is often preferred, because of the case of their ease of maintenance and genetic manipulation. They are also cheaper to house and have relatively shorter gestation times, incubation periods, and life spans. Non-human primates, such as the cynomolgus macaques (Lasmezas *et al.*, 2005) and squirrel monkey (Williams *et al.*, 2007), have been found relevant to transmission studies of human prions because they are evolutionary closer to humans and therefore could neuropathologically and physiologically model human disease more precisely. To investigate the species barrier effect on transmission of prion diseases, mouse lines expressing prion gene sequences from other species have been developed (Scott and Fraser, 1989; Telling *et al.*, 1994; Bruce *et al.*, 1997; Hill *et al.*, 1997a; Scott *et al.*, 1999; Barron *et al.*, 2001; Asante *et al.*, 2002; Kitamoto *et al.*, 2002; Taguchi *et al.*, 2003; Asano *et al.*, 2006; Asante *et al.*, 2006; Bishop *et al.*, 2006; Cancellotti *et al.*, 2006; Kobayashi *et al.*, 2007; Beringue *et al.*, 2008). It is well known that transmission of prion diseases is more efficient within species rather than between species (Bruce *et al.*, 1994). The species barrier is defined by the observation that transmission to a different species results in a significantly longer incubation period or even sometimes the absence of clinical disease (Carp *et al.*, 1994). An inverse correlation between the length of the

incubation period and the logarithm of the dose of PrP^{Sc} in the inoculum was observed and this allowed for the development of rapid bioassays (Prusiner *et al.*, 1980a; Prusiner *et al.*, 1980b; Prusiner *et al.*, 1982).

Several prion diseases (scrapie, BSE, FSE, sCJD, vCJD, kuru fCJD, FFI and one GSS variant) have been successfully transmitted to experimental animals, including rodents and non-human primates (Kimberlin and Walker, 1989; Kitamoto *et al.*, 1991; Lasmezas *et al.*, 1997; Carp *et al.*, 1998; Lasmezas *et al.*, 2001). The neuropathological diversity, such as the severity and distribution pattern of the vacuolation in mice, as well as length of incubation time have been very useful to distinguish between strains of prion agents (Bruce *et al.*, 2001).

Experimental methods for the production of transgenic mice have developed rapidly over last years, employing two distinct methods, either random genomic insertion or gene targeting (Manson and Tuzi, 2001). Mice in which *Prnp* has been experimentally disrupted (knock-out mice or $PrP^{0/0}$) are resistant to prion infection, as was proposed by the absence of replication of the prion agent in tissues and lack of clinical or neuropathological signs of disease (Brandner *et al.*, 1996a; Brandner *et al.*, 1996b). In contrast, mice overexpressing PrP have been shown to have shorter incubation periods than wild type mice inoculated with the same prion agent, providing useful models for assaying prion infectivity (Prusiner *et al.*, 1990).

The use of animal models has allowed examination of tissues during pre-clinical disease in order to examine the progression of pathological features as well as those present at terminal stage of the disease. Employing engineering of transgenic animals, it has been possible to investigate important issues of strain or species barriers, the effect of disease associated mutations, and the importance of specific

parts of the prion protein in terms of normal cellular biology and disease transmission (Kimberlin and Walker, 1979; Prusiner *et al.*, 1990; Bueler *et al.*, 1992; Prusiner *et al.*, 1993; Manson *et al.*, 1994; Prusiner and Scott, 1997; Scott *et al.*, 2000; Bishop *et al.*, 2006).

1.4.2 In vitro models for studying prion diseases – Cell cultures

Transmission studies employing wild-type and transgenic animals have been fundamental in investigating the basis of prion diseases. However, these assays tend to be long-term, very expensive and require large numbers of animals. Early attempts to propagate the prion agent *in vitro* were complicated by the surprising finding that only a very limited number of cell lines were able to replicate prions and that this property was prion strain-specific. However, when successful, the cell-based model provided a proof of principle that cells could complement or advantageously replace animal bioassays to titre prions.

1.4.2.1 Scrapie cell assay

Applications of *in vitro* models allowing the study of particular aspects of the prion disease infection process have attracted broad scientific interest and have undergone extensive development. A highly sensitive cell-based bioassay, termed scrapie cell assay (SCA), has been developed by C. Weisssmann's group (Klohn *et al.*, 2003). SCA was reported to be a quantitative measure of infectious titre, approximately as sensitive as a mouse bioassay, ten times faster, more than two orders of magnitude less expensive and suitable for automatisation (Klohn *et al.*, 2003; Bedecs, 2008; Aguzzi and Calella, 2009). In SCA, the susceptible cells are usually exposed to samples containing infectious prions, then grown until confluent and passaged. If

infection is successful, a PrP^{res} assay reveals positive cellular foci relating to the infectious titre. However, this murine system is susceptible to some, but not all murine adapted scrapie strains and has not yet been successfully applied to human prion strains or isolates (Bosque and Prusiner, 2000; Solassol *et al.*, 2003; Mahal *et al.*, 2007).

1.4.2.2 Establishment of prion infected cell cultures as a model for studying prion diseases

The initial successful attempts to establish scrapie-infected culture were accomplished in 1970 when brain explants derived from mice infected with scrapie propagated low levels of prions (Clarke and Haig, 1970). The growth and morphological characteristics of the tissue were comparable with those from uninfected mice (Field and Windsor, 1965). Employing the explant technique, the first successful cell culture termed SMB cells (scrapie mouse brain) was established *in vitro*, remaining its infectivity and this system is still in use today (Field and Windsor, 1965; Haig and Pattison, 1967; Clarke and Haig, 1970; Caspary and Bell, 1971; Birkett *et al.*, 2001; Hooper, 2002; Kanu *et al.*, 2002; Bate *et al.*, 2004a; Bate *et al.*, 2004b; Bate *et al.*, 2004c; Uppington and Brown, 2007; Bate and Williams, 2011). However, one disadvantage of this approach is the lack of uninfected controls for comparative purposes. Therefore, cells from several sources and variety of experimental systems have been used to try to establish cell cultures chronically infected with prions. The most straightforward approach, that proved to be successful for certain cell types is the direct exposure of a cell monolayer or cell suspensions to scrapie-infected brain homogenates or partly or highly purified preparations (Butler *et al.*, 1988; Race, 1991). The majority of the currently used constitutively prion-

infected cell cultures have been generated using exactly this approach. Nevertheless, repeated attempts to establish constitutively prion–infected (Race, 1991) cell lines with stable PrP^{Sc} propagation have been successful in a very small number of cell lines (Table 1.4).

1.4.2.3 Neuronal vs. non-neuronal origin of the cultured cells

Because the pathogenic isoform of the prion protein accumulates mainly in neurons which are also the targets for prion-induced neurodegeneration, the main focus has been on the development of cell cultures of neuronal origin or a neuronal phenotype. However, non-neuronal cells, for example, the recently developed scrapie infected Schwann cell cultures, could be used to investigate the peripheral route of prion invasion (Follet *et al.*, 2002; Archer *et al.*, 2004). An interesting feature of the cell lines contained in the Table 1.4 is that majority of the cell lines susceptible to chronic prion infection are of non-neuronal origin. Among the neuronal cell lines, the successful infection has been mostly achieved using mouse passaged sheep scrapie to infect rodent neuroblastoma cells (Race, 1991; Solassol *et al.*, 2003). Among these, the cells capable of continuous replication of prions are N2a (Race *et al.*, 1987; Butler *et al.*, 1988), N1E-115 (Markovits *et al.*, 1983), and C-1300 (Race *et al.*, 1987) murine neuroblastoma cell lines of common origin, derived from spontaneous tumour arising in A/J mice (Klebe and Ruddle, 1969; Markovits *et al.*, 1983) and GT1 cell line, originating from hypothalamic neurons immortalised by genetically targeted tumorigenesis in transgenic mice (Mellon *et al.*, 1990; Schatzl *et al.*, 1997; Nishida *et al.*, 2000; Arjona *et al.*, 2004). Even rat pheochromocytoma PC12 cells when differentiated to neuron-like cells can be infected with mouse prions (Rubenstein *et al.*, 1984). Mouse cholinergic septal neuronal cell line termed SN56

was also shown to be permissive to infection with several mouse-adapted prion strains (Baron *et al.*, 2006). Neuronal stem cells derived from conventional or transgenic mice were recently shown to propagate mouse-adapted prions (Giri *et al.*, 2006; Milhavet *et al.*, 2006) and moreover hippocampal-derived HpL3-4 cells isolated from PrP^{C} knock-out mouse and transfected with mouse PrP^{C} were shown to be susceptible to the mouse adapted strain 22L (Maas *et al.*, 2007). Some fibroblast cell lines (Vorberg *et al.*, 2004) and microglial cell line (MG20) established from transgenic mice overexpressing PrP^{C} were shown to be susceptible to various murine prion strains and also to mouse adapted bovine spongiform encephalopathy agent (Iwamaru *et al.*, 2007). Also cells from the peripheral nervous system such as Schwann-like cells called MSC80, were shown to replicate low levels of the RML strain (Follet *et al.*, 2002).

A recently established prion-infected cell line originating from skeletal muscle (C2C12) could be useful in investigation of prion infection of muscles observed in sheep and cervids (Dlakic *et al.*, 2007). The hamster brain HaB cell line was show to stably replicate hamster prions (Taraboulos *et al.*, 1990b).

The first example of natural TSE agent transmission (not previously adapted to rodents) occurred in the rabbit epithelial cell line RK13 which expresses ovine PrP^{C} (variant VRQ associated with a very high susceptibility (Hunter, 1997) when it was shown to successfully replicate sheep scrapie prions (Vilette *et al.*, 2001). This model also yielded the first evidence that PrP^{Sc} can propagate *in vitro* in cells of non-neuronal lineage and since then PrP^{Sc} multiplication has been demonstrated in several other non-neuronal cell lines, including fibroblasts (Vorberg *et al.*, 2004; Raymond *et al.*, 2006), microglial cells (Iwamaru *et al.*, 2007), muscle cells (Dlakic *et al.*,

2007) and a mule deer cell line were successfully infected with naturally occurring chronic wasting disease (Raymond *et al.*, 2006). The mouse neuronal CAD cell line was recently recognised as being a robust system for prion propagation in culture (Mahal *et al.*, 2007; Dron *et al.*, 2009; Gousset *et al.*, 2009).

Despite the wealth of data showing that cultured cells of a variety of species and phenotypes can propagate animal prions, evidence that human cells have been infected with a human prion agent is restricted to a single report from 1995. In this Ladogana and colleagues (Ladogana *et al.*, 1995) described their success in infecting human neuroblastoma SH-SY5Y (*PRNP* codon 129 genotype MM) cell line with 1 ml of 1% brain homogenate originating from sporadic CJD patient (*PRNP* codon 129 genotype MM). Eleven of subcloned cells were tested positive for PrP^{Sc} production by Western blot after 12 passages. Four of these clones remained PrP^{Sc} positive from the 12^{th} to 30^{th} passage, indicating persistent PrP^{Sc} propagation of human neuroblastoma cells. However, occasionally these cells were tested PrP^{Sc} negative in passages that subsequently were positive (Ladogana *et al.*, 1995) and this initial success in infecting human cells with CJD agent has not been capitalised on, most likely because the infection proved to be unstable. Interestingly, neither rabbit kidney epithelial cells RK13 expressing human PrP were permissive to infection when exposed to sporadic Creutzfeldt–Jakob disease prions (Lawson *et al.*, 2008).

1.4.2.4 Selective cell tropism of different prion strains

The cell lineages described above summarised in Table 1.4 appear to be especially susceptible to mouse-adapted prion isolates from scrapie. This is even more apparent in a view of the large number of neuronal and neural cell lines that could not be infected (Markovits *et al.*, 1983; Elleman, 1984; Butler *et al.*, 1988; Race, 1991; Chesebro *et al.*, 1993; Vella *et al.*, 2007). Interestingly, GT1 cells are susceptible to

prions other than the Chandler or RML isolates and have shown to replicate both the scrapie-derived 139A and 22L, as well as familial GSS- and sporadic CJD-derived FU and SY prions, whereas only the transfected N2a cells overexpressing PrP^{C} (N2a#58) are susceptible to infection with other isolates than Chandler or RML (Nishida *et al.*, 2000; Arjona *et al.*, 2004).

Cell term	Species of	Tissues of origin or cell type	TSE strain	Reference(s)
N2a	Mouse	Neuroblastoma	Chandler, Fukuoka-1, RML	Race *et al.,* 1987; Butler *et al.,* 1988
N2a #58	Mouse	Neuroblastoma	Chandler, 139A, 22L	Nishida *et al.,* 2000
C-1300	Mouse	Neuroblastoma	Chandler	Race *et al.,* 1987
N1E-115	Mouse	Neuroblastoma	Chandler	Race *et al.,* 1987; Ostlund *et al.,* 2001
MNB	Mouse	Neuroblastoma	Chandler	Race *et al.,* 1987
HaB	Hamster	Hamster brain cells	Hamster strain	Tarabolous *et al.,* 1990
SN56	Mouse	Cholinergic septal neuronal	Mouse adapted scrapie, 22L	Hammond *et al.,* 1990; Magalhaes *et al.,* 2005
SH-SY5Y	Human	Neuroblastoma	sCJD	Ladogana *et al.,* 1995
GT 1-7	Mouse	Hypoth. neur. cells	22L, Chandler, 139 A	Schatzl *et al.,* 1997; Nishida *et al.,* 2000
GT 1	Mouse	Hypoth. neur. cells	Sy-CJD, FU-CJD, Fukuoka	Arjona *et al.,* 2004; Arima *et al.,* 2005
DRG	TgovMouse	Dorsal root ganglia	Natural sheep scrapie	Archer *et al.* , 2002
MovS	TgovMouse	Schwann-like cells	Scrapie	Archer *et al.* , 2002
MovS6//S2	TgovMouse	Schwann-like cells	PG127	Archer et al., 2004
MSC-80	Mouse	Schwann cells	Chandler	Follet *et al.,* 2002
T98G	Human	Glioblastoma	natural conversion	Kikuchi *et al.,* 2004
CGN 338, CAS 358	TgovMouse	Astrocyte/neurons	Scrapie, 139A, ScMov	Cronier, 2004
NSC	Mouse	Neuronal stem cells	RML, 22L	Giri *et al.* , 2006; Milhavet *et al.* , 2006
HpL3-4	Mouse	Hippocampal cells	22L	Maas *et al.,* 2007
SMB	Mouse	Brain cells (mesodermal origin)	Chandler	Clarke *et al.,* 1970; Birkett *et al.,* 2001
L fibroblast	Mouse	Fibroblast	Chandler	Clarke *et al.,* 1976
L929	Mouse	Fibroblast	22L,ME7 & RLM	Vorberg *et al.,* 2004
L929, NIH/3T3	Mouse	Fibroblast	22L	Vorberg *et al.,* 2004
L23	Mouse	Unspecified	C-506	Cherednichenko *et al.,* 1985
NS1	Mouse	Spleen hybridoma with NS1	Chandler	Elleman, 1984
PC12	Rat	Pheochromocytoma	139A, ME7	Rubenstein *et al.,* 1984
Glial cells	Rat	Glial cells (Gasserian ganglion)	Chandler	Roikhel *et al.,* 1984
RK-13	Rabbit	Kidney epithelial	Natural sheep scrapie	Vilette *et al.,* 2001
moRK13	Rabbit	Kidney ephitelial RK13	Fukuoka-1, Chandler, M100	Corageot *et al.,* 2008; Vella *et al.* , 2007
voRK13	Rabbit	Kidney ephitelial RK13	Vole-adapted BSE	Corageot *et al.,* 2008
MG20	Mouse	Microglial	Chandler, ME7, mouse BSE	Iwamaru *et al.,* 2007
C2C12	Mouse	Myoblasts	22L	Dlakic *et al.,* 2007
MDB	Deer	Fibroblast-like	Chronic waisting disease	Raymond *et al.,* 2006
Rov	Mouse	Kidney epithelial cells	PG127, LA404	Vilette *et al.,* 2001; Sabuncu *et al.* , 2003
CAD	Mouse	Catecholaminergic neuronal	139A, 22L	Mahal *et al.,* 2007; Dron *et al.,* 2009

Table 1.4: Cell culture models established to study prion diseases

GT1 cells have important advantages over the use of N2a cells, particularly for studying cytopathological effects induced by prion infection, since they express approximately eight times higher levels of endogenous PrP^{C} (Nishida *et al.*, 2000) and consequently they are much more susceptible to prion infection than N2a cells. Only 2% or less of the prion exposed N2a cells become infected and only low levels of PrP^{Sc} are produced in the culture. These cultures frequently loose their infected

status after 10-15 passages (Race *et al.*, 1987; Butler *et al.*, 1988; Bosque and Prusiner, 2000).

To obtain chronically prion-infected N2a cultures that produce sufficient levels of PrP^{Sc}, N2a cultures have to be subcloned prior to prion exposure. Subcloning can provide lines with 80-90% of prion-infected N2a cells (Bosque and Prusiner, 2000; Nishida *et al.*, 2000; Enari *et al.*, 2001) and these have been shown to be very useful in studying the cell biology of prion replication, subcellular localisation of PrP^{Sc} and the kinetics of PrP^{Sc} formation (Borchelt *et al.*, 1990; Taraboulos *et al.*, 1990b; Caughey and Raymond, 1991; Taraboulos *et al.*, 1995; Gorodinsky and Harris, 1995) as well as in the development of inhibitors of PrP^{Sc} formation and antiprion prophylaxis (Caughey and Race, 1992; Winklhofer and Tatzelt, 2000; Supattapone *et al.*, 2002). The SMB cells that originated from Chandler strain-infected mouse brain culture (Clarke and Haig, 1970) were shown to be able to be "cured" of prion infection by treatment with pentosan sulphate and were then re-infected with other mouse-adapted prion strains (Birkett *et al.*, 2001; Kanu *et al.*, 2002). The L929 murine fibroblast cell line was shown to be susceptible to 22L, ME7 and RML prion strains, whereas the MG20 microglial cell line is susceptible to ME7, Chandler and the mouse-adapted BSE agent. The RK13 cell line expressing mouse PrP^{C} was shown to be susceptible to several murine prion strains (Courageot *et al.*, 2008). However, the observed cell tropism could be illustrated for the ME7 strain which could not be replicated in N2a or GT1 cell lines (Bosque and Prusiner, 2000; Klohn *et al.*, 2003), although the SN56 (Baron *et al.*, 2006), L929 (Vorberg *et al.*, 2004), and MG20 (Iwamaru *et al.*, 2007) were shown to propagate this prion strain. The molecular and cellular basis of these effects is not yet understood.

1.4.2.5 Prions propagated in cell cultures retain their strain characteristics

Several murine strains (22F, Chandler, Fukuoka, SY) propagated in N2a, GT1 or SMB cell cultures (Birkett *et al.*, 2001; Arjona *et al.*, 2004; Arima *et al.*, 2005) have been shown to retain their biological characteristics (incubation time, vacuolisation, clinical signs) after passage through cultured cells. Also, biological characterisation of sheep, mouse, and vole prion strains replicated in cell line expressing ovine, mouse or vole PrP^{C} (RK13 cell line), suggests that these strains are not modified by replication in non-neuronal cells (Courageot *et al.*, 2008). The PrP^{res} banding patterns of strains generated by infected cell cultures do sometimes differ from those generated in the brain (Caughey *et al.*, 1991; Nishida *et al.*, 2000; Vilette *et al.*, 2001; Archer *et al.*, 2004; Arima *et al.*, 2005; Milhavet *et al.*, 2006) but return to their original pattern upon animal inoculation (Archer *et al.*, 2004; Arima *et al.*, 2005). Therefore, the biological properties of the strain are not modified after propagation in cultured cells. Interestingly and importantly, although brain and cell generated PrP^{res} patterns are different, strain specific banding pattern differences observed for prion brain PrP^{res} have also been reported after serial propagation of certain agents in certain cultured cells (Birkett *et al.*, 2001; Arima *et al.*, 2005; Iwamaru *et al.*, 2007; Courageot *et al.*, 2008).

Attempts to study the very early events during infection of cultured cells have proved to be rather difficult because a) only a few cell culture systems have been identified to be susceptible to prion agents, b) newly formed PrP^{Sc} is difficult to distinguish from PrP^{Sc} present in the inoculum, and c) the amount of PrP^{Sc} produced in susceptible cell lines at early stage of infection is often very low (Vorberg *et al.*, 2004).

One of the principal objectives in establishing prion-infected cell cultures has been to look for the cythopathological and morphological consequences of the prion infection. However, a striking feature was the lack on any obvious signs of cell death (Clarke and Haig, 1970). Notwithstanding that the establishment of scrapie infected cell lines or cell lines expressing mutant PrP^{C} linked to some hereditary prion diseases has been crucial for the understanding of the biogenesis and metabolism of PrP^{Sc}, the handicap in using scrapie-infected cell cultures to study molecular mechanism underlying prion-induced neurodegeneration is the lack of obvious signs of neurotoxicity in these cells. The discrepancy between *in vivo* and *in vitro* PrP^{Sc} neurotoxicity could be due to the transformed phenotype of the currently available cell culture models, thus masking the PrP^{Sc} neurotoxicity that may occur only in finally differentiated cells, such as postmitotic cells of the adult CNS. This could explain why ScGT1 cells, owning more differentiated neuronal phenotype than ScN2a and ScN1E-115 cells, do exhibit certain signs of neurodegeneration, whereas ScNa2 and ScN1E-115 cells do not.

1.4.3 In vitro models for studying prion diseases – Cell-free conversion systems

The prion "protein-only" hypothesis proposes that the fundamental event in prion diseases is the conversion of the normal cellular prion protein (PrP^{C}) into the misfolded disease-associated protein (PrP^{Sc}) and that this conversion process is PrP^{Sc}-dependent and requires the physical interaction of PrP^{C} with PrP^{Sc} (Prusiner, 1998). Studies using model systems have also suggested that host-encoded factors other than PrP^{C} may be required to propagate prions *in vitro* and *in vivo* (Telling *et al.*, 1995; Saborio *et al.*, 1999; Stephenson *et al.*, 2000; Lloyd *et al.*, 2001).

Furthermore, the restricted range of neuronal and non-neuronal cell types that are susceptible to infection with prions also indicates the existence of prion propagation cofactors (Raeber *et al.*, 1999; Bosque and Prusiner, 2000; Enari *et al.*, 2001).

Although PrP conversion in cultured cells and animal models is clearly possible, it has been difficult to reproduce the process *in vitro* in a cell-free system. To establish a cell-free model system that supports misfolding of PrP, a number of assays have been devised (Ryou and Mays, 2008). The molecular requirements and recapitulation of the PrP^{C} to PrP^{Sc} conversion process have been studied more fully employing *in vitro* systems implementing chemical and physical treatments of PrP that involve denaturation producing a form of PrP that shares some of the properties of PrP^{Sc} (Jackson *et al.*, 1999).

1.4.3.1 Protein misfolding cyclic amplification

The first template dependent cell-free PrP conversion system was devised by B. Caughey and colleagues, reviewed by (Jones *et al.*, 2011), but an invaluable technical innovation was developed by C. Soto and co-workers in a the form of a technique called protein misfolding cyclic amplification (PMCA), in which PrP^{Sc} and also infectivity are both amplified (Saborio *et al.*, 2001; Castilla *et al.*, 2005a; Saa *et al.*, 2006; Castilla *et al.*, 2006). PMCA recapitulates the prion protein conversion process in cell-free conditions and it is a cyclic process consisting of two alternating phases - incubation interrupted by sonication at regular intervals leading to accelerated prion replication (Saborio *et al.*, 2001; Soto *et al.*, 2002). This suggests that during the first phase, a sample containing minute amounts of "seed" PrP^{Sc} recruits monomeric PrP^{C} "substrate" molecules and converts it into PrP^{Sc}.

The sonication step based on ultrasound-induced fragmentation breaking down the

PrP^{Sc} aggregates into smaller subunits, each of which acts as a new template for recruiting more PrP^{C}, which is subsequently converted into PrP^{Sc} (Figure 1.10). Potentially exponential amplification is achieved by each successive cycle limited only by the availability of substrate and co-factors (Saborio *et al.*, 2001; Bieschke *et al.*, 2004).

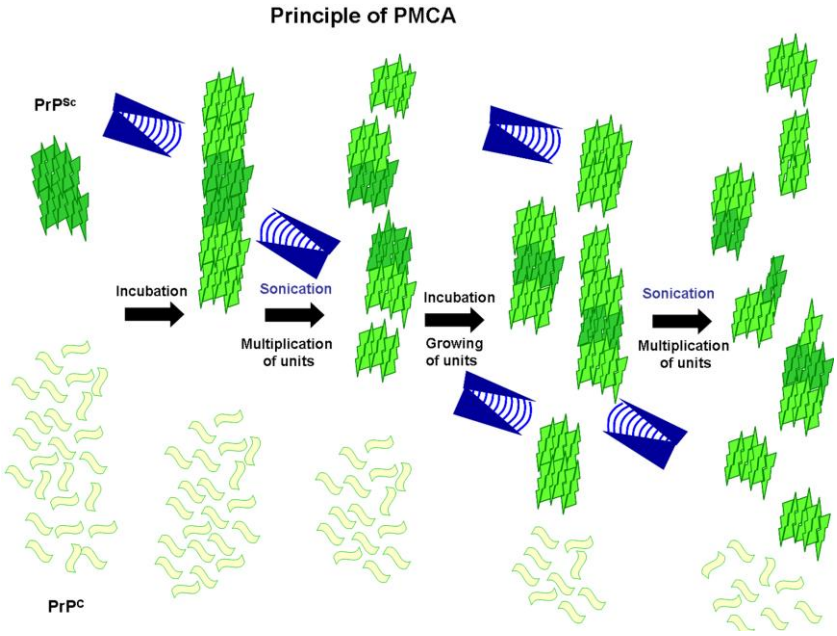


Figure 1.10: Diagrammatic scheme of the principle of PMCA reaction
PMCA consists of a phase when a minute amounts PrP^{Sc} multimers (darker green) in the presence of excess of PrP^{C} monomers (ligh yellow) act as a seed for PrP^{C} to PrP^{Sc} conversion. The newly converted polymers (lighter green) are then subjected to additional cycles of sonication-providing more seeds for PrP^{C} to PrP^{Sc} conversion and subsequent incubation-providing amplification of newly converted PrP^{Sc} units. The PrP^{Sc} amplification process is limited by availability of PrP^{C} substrate. Figure modiefied from (Catilla *et al.*, 2006).

PMCA was originally established employing brain material and latter partially purified and recombinant PrP^{C} (Atarashi *et al.*, 2007; Kim *et al.*, 2010). PrP^{Sc} generated by PMCA was shown to be infectious in wild-type animals (Castilla *et al.*, 2005a) and can be indefinitely propagated preserving properties of the original PrP^{Sc} (Castilla *et al.*, 2006; Shikiya *et al.*, 2010).

Furthermore, PMCA was shown to be useful in identifying of conditions and

cofactors influencing PrP conversion (Deleault *et al.*, 2003; Lucassen *et al.*, 2003; Nishina *et al.*, 2006; Deleault *et al.*, 2007; Geoghegan *et al.*, 2007; Murayama *et al.*, 2007; Kim *et al.*, 2009; Mays *et al.*, 2009; Mays and Ryou, 2010; Abid *et al.*, 2010), and in detecting PrP^{Sc} from biological samples of humans and animals (Soto *et al.*, 2005; Castilla *et al.*, 2005b; Atarashi *et al.*, 2007; Kurt *et al.*, 2007; Jones *et al.*, 2007; Thorne and Terry, 2008; Jones *et al.*, 2009; Haley *et al.*, 2009; Rubenstein *et al.*, 2010; Tattum *et al.*, 2010a; Tattum *et al.*, 2010b). Supattapone and co-workers identified the minimal components (PrP^{C}, co-purified lipids and single stranded polyanionic molecules) for *de novo* generation of infectious PK resistant PrP^{Sc} from normal hamster brain using PMCA. Inoculation of these reaction products into healthy hamsters caused prion disease (Deleault *et al.*, 2007), strongly support the prion hypothesis.

Another recently developed method - involving seeded conversion of the α-helix-rich form of bacterially expressed recombinant PrP^{C} to a β-sheet-rich amyloid fibrillar form - is termed Quaking-induced conversion (QuIC) (Atarashi *et al.*, 2008; Wilham *et al.*, 2010). QuIC and its even more recently introduced real-time variation (RT-QuIC) are promising diagnostic tests for prion diseases because their sensitivity may be sufficient to detect the low levels of PrP^{Sc} thought to occur in body fluids such as cerebrospinal fluid (CSF) and blood (Atarashi *et al.*, 2011; Orru *et al.*, 2011).

1.5 PRIONS AND PUBLIC HEALTH

1.5.1 Human-to-human routes of prion transmission

One implication of the humanised transgenic mice experimental models (Telling *et al.*, 1995; Manson and Tuzi, 2001; Asante and Collinge, 2001; Weissmann and

Flechsig, 2003; Bishop *et al.*, 2006; Beringue *et al.*, 2008) findings is that a significant number of individuals in the UK population may be infected with vCJD but are asymptomatic and present a risk of disease transmission to others by, for example blood transfusion. In the case of the four known cases resulting from prion transmission by blood transfusion the incubation time periods from the donation to development of clinical signs of vCJD in the donors were 40 months (Llewelyn *et al.*, 2004), 20 months (Wroe *et al.*, 2006), and 17 months (HPA, 2007; http://www.cjd.ed.ac.uk/TMER/TMER.htm). This re-affirms the suggestions that the vCJD pre-clinical phase is lengthy and also that during that time period the asymptomatic individual is able to transmit the disease via tissues and fluids.

Blood transfusion is not the only means of accidental transmission of infectious prions. Historically, corneal transplantation, neurosurgical instrument re-use, dura mater grafting and growth hormone therapy have all been associated with iatrogenic transmission of CJD (Brown, 1990; Collins *et al.*, 2004). More recently concern has been expressed about surgery (Ramasamy *et al.*, 2003; Mabbott and Turner, 2005) and dentistry (Bennett *et al.*, 2007) as a possible routes of vCJD iatrogenic transmission. The potential for future stem cell therapies to transmit prion disease has received comparatively little attention. Most current culture systems supporting human embryonic stem cell self-renewal and differentiation rely both on direct and indirect exposure to animal and human cells, and on the use of cell products such as purified human proteins or bovine serum (De Sousa *et al.*, 2006). These components can represent a potential risk of prion infection as indeed may the cells themselves, depending on their origin. Given the precedent of iatrogenic CJD, it seems prudent to

consider the potential risk of prions transmission by the newly emerging cell therapies.

1.6 AIMS

A major problem in human prion disease research is the lack of a suitable experimental model system in which to study the molecular mechanisms involved in cellular susceptibility to infection and the processes involved in prion replication. This is largely due to the lack of well characterised cultured human cells that are susceptible to infection with human prions, such as those involved in Creutzfeldt-Jakob disease.

Follicular dendritic cells (FDC) are known to support prion replication *in vivo*, for example in vCJD. Thus this thesis sought to determine whether a human follicular dendritic cell-like (FDC-like) cell line (termed HK) had all of the required components to support prion replication and to examine the susceptibility of these cells to prion infection and their ability to support prion propagation *in vitro*. The mechanisms of exogenous PrP^{Sc} internalisation, intracellular trafficking and its fate within the cell were subsequently examined.

Given the known potential for iatrogenic transmission of prion diseases such as CJD, a parallel series of experiments considered the susceptibility to prion infection of human embryonic stem cells (hESC) by characterising their genetics and expression of the normal cellular prion protein and by examining their response to acute exposure to prions from a range of sources.

2. MATERIALS AND METHODS

2.1 SAFETY AND MANIPULATION REGULATIONS

All brain tissue sampling procedures were conducted in the class 1 microbiological safety cabinet in the National CJD Research & Surveillance Unit (NCJDRSU) category 3* High Risk Laboratory. The Advisory Committee on Dangerous Pathogens (ACDP) classifies TSE agents in various species as hazard group 3 (http://www.dh.gov.uk/ab/ACDP/index.htm). Precautions included wearing standard splash proof gown and disposable high-risk over-gown, protective footwear, protective eyewear and double gloves. Particular care was taken at steps that had the potential to cause aerosols. Sealed rotors were used for all centrifugation steps and were only opened inside the microbiological safety cabinet.

The prion agents are resistant to inactivation by conventional chemical and physical decontamination methods and exhibit exceptional stability against such treatments. Prion decontamination recommended by the ACDP and as required by the Laboratory code of practice in NCJDRSU is to incubate infective material with 2 M sodium hydroxide (NaOH) for minimum 1 hour before disposal.

Operations involving cell culture genotyping were performed in the class 2 microbiological safety cabinet.

The entire cell culture manipulation has been conducted in by a UV radiation pre-sterilised class 2 microbiological safety cabinet in the category 3* high risk laboratory. All reagents, tools and techniques used throughout were under aseptic conditions. Handling cell culture was largely a matter of containment and avoidance of aerosols. This is justified by two aspects: first, aseptic technique (protecting the

cultures from the researcher) and second, safety (protecting researcher from the cultures and brain tissue homogenates). Steps were taken to avoid spills and use of secondary containment when transporting cell culture flasks/chamber slides between hood, incubator, microscope, etc. Stem cells from MRC Centre for Regenerative Medicine were transported to the NCJDRSU by private car or taxi as well as the sealed slides containing prion exposed cells for confocal microscope analysis from NCJDRSU to the Neuropathogenesis division of the The Roslin Institute & Royal (Dick) School of Veterinary Sciences. The samples were transported under the regulations for transporting biological substances of category B assigned to UN 3373 (http://www.docs.csg.ed.ac.uk/Safety/bio/guidance/transport/summary_catB.pdf) in accordance with ADR P650 packaging instructions.

2.2 CELL CULTURES

2.2.1 HK cell line

The follicular dendritic cell (FDC)-like HK cell line was a kind gift to the National CJD Research & Surveillance Unit from Dr. Yong Sung Choi, Cellular Immunology Laboratory, New Orleans, USA. This HK cell line was originally established from human tonsils to investigate the functional role of FDCs in germinal centres of lymphoid follicles. The isolation is described in Kim *et al.,* 1994. In short, freshly obtained tissue from human tonsils was enzymatically digested and subjected to Percoll gradient centrifugation (Tsunoda *et al.*, 1990; Clark *et al.*, 1992). Cells with densities less than 1.050 g/ml were plated on tissue culture dishes. Non-adherent lymphoid cells were removed. After growing for two weeks the cultures were morphologically homogenous, composed of proliferating non-phagocytic adherent

cells with long cytoplasmic protrusions and were termed HK cells. These cells were established in the absence of exogenous cytokines (GM-CSF), growth factors or EBV-transformation and continue growing over a 180 days without changes in viability. Surface phenotype markers of these cells were periodically examined by fluorescent microscopy (Kim *et al.*, 1994).

HK cells at 1-2 days of culturing expressed characteristic FDC antigens including DRC-1, HJ2, CD21 and surface Ig. Although the FDC-specific marker, DRC-1 antigen, was initially expressed by HK cells, it was not detectable after 3 days in culture. However expression of another FDC-specific marker, HJ2, was retained. Furthermore, HK cells promoted B cell proliferation. The pattern of HK cells surface markers expression corresponded to reported FDC phenotype (Schriever *et al.*, 1989; Petrasch *et al.*, 1990).

Besides of resembling FCD cells the HK cells supposedly also shared some phenotype and morphologic characteristics of fibroblasts (Kim *et al.*, 1994). The requirements of growth factors, growth kinetics and cytokine production of HK cells was therefore compared with age-matching human skin and human foetal lung fibroblasts. However, the analysis of markers suggested that the HK cells were functionally and phenotypically distinct from fibroblasts (Kim *et al.*, 1994).

Subsequent studies showed that the FDC-like HK cells contribute a favourable environment for the growth of follicular lymphoma cells (FLK-1) when cultured together (Kagami *et al.*, 2001). FDCs are believed to support lymphoma cell growth (Stein *et al.*, 1982; Petrasch *et al.*, 1992; Choe *et al.*, 2000), and this observation has been used to support the proposed FDC-like phenotype of HK cells. Nevertheless, it is important to note that several B-lymphoma cell lines have been established from

follicular lymphoma not requiring FDCs for their growth (Doi *et al.*, 1987; Amakawa *et al.*, 1990; Matsumura *et al.*, 1990; Mohammad *et al.*, 1993; Sambade *et al.*, 1995).

2.2.1.1 Establishing HK cell cultures from frozen stocks

A cryogenic vial (Corning, UK) containing 1 ml frozen cell culture stock in 100% foetal calf serum (FCS, Invitrogen, UK) was removed from -150 °C cryogenic freezer (Revco Ultima II, Kendro Laboratory products, USA) and the suspension was thawed immediately. The cell suspension was gently dispersed in 9 ml of 37 °C pre-heated RPMI 1640 culture medium (Invitrogen, UK) containing 1% Penicillin/Streptomycin/Amphotericin antibiotics/antimycotic cocktail (100X stock contains 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin and 25 µg/ml of amphotericin B as Fungizone® Antimycotic in 0.85% saline, Invitrogen, UK) in a 15 ml Falcon tube (Fisher Scientific, UK) and then with gentle trituration transferred into two T25 cell culture flasks (Corning, UK). Cultures were incubated at 37 °C in a 5% CO_2 cell culture incubator (Binder GmbH, Germany).

2.2.1.2 Routine cultivation of HK cells

The HK cells were routinely cultured in complete medium consisted of RPMI 1640 culture medium supplemented with 10% FCS and Penicilin/Streptomycin/Amphotericin antibiotics in T25 flasks and maintained at 37 °C in a 5% CO_2 cell culture incubator. Full medium changes were performed every other day with pre-warmed (37 °C) medium.

2.2.1.3 Passaging HK cell cultures

Passaging the HK cells was performed when the culture reached 80-90% confluency (roughly every ~10 days) at ratio 1:5. Briefly, medium was aspirated and disposed of into 2 M sodium hydroxide (NaOH). The cells were rinsed twice with 5 ml

1X Hank's Balanced Salt Solution (HBSS, Invitrogen, UK). 1 ml of 0.05% Trypsin-EDTA (Invitrogen, UK) was added into the flask with cultures. Trypsin does cleave proteins bonding of the cultured cells to the dish and thus facilitating cell's detachment. The process was monitored using an Olympus differential interference contrast cell culture microscope with reflected fluorescence system for CKX41 equipped with Olympus CAMEDIA digital camera C-5060 wide zoom, JVC colour video monitor TM-QA14PN-K, Olympus power supply U-RFLT50. Data were exported using CAMEDIA Master 4.1 software. After detachment 3 ml of complete culturing medium was added and the cell's suspension was transferred into a sterile 15 ml Falcon tube. Cells were collected by centrifugation at 884 rpm for 10 minutes (rotor 1180, Sigma 3-16K centrifuge, DJB Labcare, UK). The supernatant was aspirated and disposed of. The cell pellet was gently resuspended in complete culture medium. The cell suspension was transferred into fresh T25 cell culture flask.

2.2.1.4 Cryopreservation of HK cell cultures

The cell cultures selected to be frozen down as a stocks were detached from individual culture flasks with Trypsin-EDTA (as described in section 2.2.1.3). After centrifugation the cell pellet from one near confluent T25 flask was gently resuspended in 1 ml of 100% FCS, transferred into cryogenic vial and immediately frozen down at -150 °C.

2.2.1.5 Plating HK cells for immunocytochemistry studies

For studies involving confocal microscopy, cells were detached from the T25 flask with Trypsin-EDTA (as described in section 2.2.1.3) and plated onto chemically pre-coated 4-welled glass chamber slides Lab-Tek® II – CC^{2TM} (Nalgene Nunc International, part of Thermo Fisher Scientific, UK) one day before exposure to brain

spiked medium. These chamber slides are pre-treated by manufacturer with chemically modified glass growth surface with a positive charge that mimics Poly-D-Lysine to facilitate better attachment of the cell to the glass surface.

2.2.2 Human embryonic stem cell lines

The human embryonic stem cells (hESC) used in this study originated and from Dr. Paul De Sousa's laboratory in the MRC Centre for Regenerative Medicine, Edinburgh, UK. These cells were originally isolated as a part of a strategy for hESC derivation from clinically failed eggs whose developmental potential was rescued by parthenogenetic activation. Another goal of that project was to accomodate strategies overcoming reliance on some of the most poorly defined and path-critical factors associated with hESC derivation. These are for example an animal immune complement to isolate embryo inner mass, animal sourced serum products and feeder cells helping to sustain hESC growth and attachment.

Seven new hESC lines (RH1, RH3, RH4, RH5, RH6, RH7, and RCM-1) were isolated. The RH1-7 lines were derived by outgrowth from whole blastocyst on an extracellular matrix substrate of purified human laminin transitionally relying on mitotically inactivated human dermal fibroblast (HDF) feeder cells (Fletcher *et al.*, 2006) using HDF conditioned medium supplemented with a bovine-sourced serum replacement (bSRM, (Martin *et al.*, 2005)).

The RCM-1 (i.e., Roslin Cells Manchester-1) was derived from a failed-to-fertilize inseminated egg recovered by parthenogenetic activation - as a demonstration of alternative strategy to generate normal hESC lines from clinically failed eggs to minimize the potential conflict with donor reproductive interest to conceive (De

Sousa *et al.*, 2009). This pluripotent hESC line is comparable to hESC lines derived from surplus of infertility treatment embryos and is deposited in the UK Stem Cell Bank for access by the research community. When the RCM-1 line was compared with other diploid female hESC lines previously isolated from supernumerary IVF blastocysts by the group of P. De Sousa, namely RH1 and RH3 (Fletcher *et al.*, 2006), the initial comparison (ISCI, 2007) of these lines showed high overall similarity as expected for cells of the same type.

All three lines were isolated on feeder cells with two (RCM-1 and RH1) in HDF conditioned Dulbecco's minimal essential medium (DMEM) and one (RH3) in a chemically defined medium, X-Vivo10 (Fletcher *et al.*, 2006). All three lines generally expressed the anticipated panel of pluripotency markers, including NANOG, Oct-4, Sox-2, Dnmt3b, Lin28, CD 9, and others while uniformly exhibiting low levels of other markers (i.e., NR5A2, Noggin, FoxD3, and FGF5) (De Sousa *et al.*, 2009). However the analysis also revealed that RCM-1 and RH1, which were isolated under identical culture conditions, were more closely matched in their expression of undifferentiated markers than RH1 and RH3, which originated from siblings embryos and thus were genetically related (De Sousa *et al.*, 2009). This indicated that the methods of hESC line isolation and culture conditions may perhaps have a proportionately greater effect on gene expression than genetic identity. Because of their exclusive resemblance the RCM-1 and RH1 cell lines were chosen as a cell culture study models for this project.

2.2.2.1 Matrigel coating of flasks and wells

A primary aliquot of matrigel (Becton Dickinson Biosciences, UK) was thawed at 4 ^{o}C for at least 2 hours to avoid formation of a gel. Then the matrigel was diluted at

a ratio 1:30 with a cold Knock Out Dulbeco's Modified Eagles Medium (Invitrogen, UK) using a pipette. Matrigel suspension was added to well or flask (1 ml/6 well plate, 0.25 ml/4 welled chamber slide) and incubated overnight at 4 °C. These prepared flasks or wells were stored at 4 °C and could be use within a time period of 1 week. Before use the matrigel pre-coated cell culture container was warmed to RT and matrigel was aspirated immediately prior adding the cell culture suspension to the well.

2.2.2.2 Routine cultivation of hESC cultures

The hESC were routinely cultured in matrigel-coated tissue culture plates (Corning, UK). Cultures were maintained in an incubator at 37 °C, 5% CO_2 in air and 100% humidity. The culture medium was a Human Dermal Fibroblast Conditioned Medium - HDF-CM+, consisting of: Knock Out Dulbeco's Modified Eagles Medium (KO-DMEM, Invitrogen UK), 20% Knock Out Serum Replacement (KOSR, Invitrogen, UK), 0.1 mM MEM-non-essential amino acids (Invitrogen, UK), 1.5 mM L-glutamine (Invitrogen, UK), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, UK), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen, UK) and basic fibroblast growth factor (bFGF, Peprotech, UK) at final concetration 4 ng/ml. This medium was conditioned for 24 hours using inactivated human fibroblasts (hbFGF). The medium was stored frozen. Before use it was thawed and further supplemented with 4 ng/ml bFGF and 1.5 mM L-glutamine and filtered. Full medium changes were performed daily with water-bath pre-warmed (37 °C) medium.

2.2.2.3 Passaging of hESC cultures

The hESC were passaged when the colonies were large, and began to merge and had centres that were dense and phase-bright when compared to the edges. Morphology

was best viewed under low magnification using a differential interference contrast microscope.

Depending on the size and density of seeded aggregates, cultures were typically passaged at a ratio 1:2, usually every 5-7 days, by wash with PBS (Sigma-Aldrich, UK), treatment with 200 units/ml collagenase IV (Invitrogen, UK) in KO-DMEM, followed by incubation at 37 °C for around 5 minutes, until the edges of the colonies began to lift. The collagenase IV was then aspirated, the cells washed again with PBS and conditioned medium + hbFGF was added. The cells were then scraped (Cell scraper, Costar®, Corning, UK) and transferred into a 15 ml Falcon tubes. Gentle trituration was performed to break up colonies, and the cells were plated into new matrigel-coated (section 2.2.2.1) wells. The conditioned medium + hbFGF was added to each well and wells were gently agitated to allow even distribution of cells in a cell culture well.

2.2.2.4 Cryopreservation of hESC cultures

Cells from hESC cultures selected to be frozen down as a stocks were detached from plates by the method described previously in the section 2.2.2.3. After the cell suspension was transferred to a 15 ml Falcon tube, cells were collected by centrifugation at 1,000 rpm for 5 minutes. Next, the supernatant was aspirated and disposed of, and the cell pellet was gently re-suspended in 4 °C freezing solution CryoStorTM CS10 (Cell Cryogenics Ltd, UK). The cell suspension was transferred into pre-chilled cryogenic vials, frozen down at -80 °C overnight or longer and then transferred to liquid nitrogen storage.

2.2.2.5 Plating hESC cultures for immunocytochemistry studies

For immunocytochemistry assays the hESC were plated on glass chamber slides (Lab-Tek® II, Nalgene Nunc International, part of Thermo Fisher Scientific, UK), which were coated with mixture composed of 5% matrigel (Becton Dickinson Biosciences, UK) and 95% Hyaluronic acid (Sigma-Aldrich, UK) one day before plating. This base was used to minimise background and non-specific sticking of the brain homogenate and antibodies to the matrix. Cells were detached from plates (as described previously in section 2.2.2.3) and plated on chamber slides at a density 10^4- 10^5 cells/cm^2 one day before exposure to brain spiked medium.

2.3 BRAIN MATERIALS

2.3.1 Brain tissue selection criteria

2.3.1.1 Human brain tissues used throughout the study

Frozen brain material, from autopsy-proven, neuropathologically well characterised cases of vCJD, $iCJD^{GHT}$, sCJD were selected for the studies. A non-CJD (AD) and non-CJD World Health Organisation (WHO) (Minor *et al.*, 2004) standard cases were used as controls. The basic informations of the tissues are summarised in Table 2.1. All tissues were handled exclusively in the category 3* containment facility according to stringent health and safety protocols. All samples had consent for use in research. All samples were taken from the temporal cortex (TC). Tissue homogenates (10% w/v) were stored at -80 °C.

human						
TYPE	***PRNP* CODON 129 GENOTYPE**	**PrP^{res} TYPE**	**BRAIN REGION USED FOR STUDY**	**DURATION (months)**	**GENDER**	**AGE AT ONSET**
vCJD	MM	2B	TC	8	F	31
$iCJD^{GHT}{}_1$	VV	2A	TC	5	M	27
$iCJD^{GHT}{}_2$	VV	2A	TC	5	M	25
$iCJD^{GHT}{}_3$	MV	2A	TC	7	M	29
$sCJD_1$	MM	1	TC	4	F	86
$sCJD_2$	MM	1	TC	24	F	57
$sCJD_3$	VV	2A + 1	TC	5	M	74
AD	MM	not appl.	TC	228	M	44
non-CJD (WHO st.)	MM	not appl.	TC	not avail.	M	68

Table 2.1: Human brain tissues used in the study

2.3.1.2. Animal brain tissues used throughout the study

The study also used animal brain materials. The basic informations of the tissues are summarised in Table 2.2 and 2.3.

The cattle brain tissue was a generous gift from the VLA Biological Archive (http://vla.defra.gov.uk/services/ser_tse_archive.htm). The positive BSE cases were field suspects that had been identified through passive surveillance. These have been transported to VLA Weybridge for cull and extended post mortem examination. The

bovine (BSE negative) sample came from a known source with limited or no exposure to BSE, reared under controlled conditions and acquired as an additional tissue for the VLA archive.

animal						
TYPE	**SPECIES**	**BREED**	***PRNP* CODON 129 GENOTYPE**	**CONTROL STATUS**	**TSE STATUS**	**BRAIN REGION USED FOR STUDY**
bovine BSE_1	bovine	friesian cow	MM	BSE suspect	positive	brain stem
bovine BSE_2	bovine	friesian cow	MM	BSE suspect	positive	brain stem
bovine negative	bovine	friesian cow	MM	BSE-non-exposed control	negative	brain stem

Table 2.2: Bovine brain tissues used in the study

Gene-targeted *PRNP* humanised transgenic mice were used in this study. These unique mice lines were developed by the Neuropathogenesis Division of the Roslin Institute, Edinburgh, (Bishop *et al.*, 2006), using a methodology called gene targeting. The method involves direct replacement of mouse prion protein *Prnp* open reading frame for an exogenous human *PRNP* sequence. All three variations of the human *PRNP* polymorphisms at the codon 129 (MM, MV, and VV) were established in three Ola129 inbred mice lines with an identical genetic background. The human *PRNP* sequence is therefore under the expression control of the host, and encodes the human prion protein (Bishop *et al.*, 2006).

This study employed the humanised transgenic mice line Ola129 carrying the human *PRNP* at codon 129 MM genotype (Hu MM Tg) as the substrate for the PMCA reaction. Hu MM Tg substrate sample was either seeded with PrP^{Sc} originating from vCJD brain material (a positive control for amplification) or un-seeded (a negative control) in PMCA reactions alongside the cell culture test samples.

animal						
TYPE	**SPECIES**	**LINE**	***PRNP* CODON 129 GENOTYPE**	**CONTROL STATUS**	**TSE STATUS**	**BRAIN REGION USED FOR STUDY**
Hu tg mouse	murine	129 Ola	MM	TSE-non-exposed control	negative	half brain

Table 2.3: Humanised transgenic mice tissue used in the study

2.4 ANTIBODIES AND MARKERS USED THROUGHOUT THE STUDY

Table 2.4 summarises primary and secondary antibodies and markers used for experimental investigations in the studies described in this thesis.

ID	Description	Epitope	Type	Application	Concentration	Provided
Primary mAb's						
3F4	anti-prion protein mAb	human a.a.109-112	mouse IgG2a	WB ICC	0.2 µg/ml 4 µg/ml	Millipore, UK
6H4	anti-prion protein mAb	human a.a. 144-152	mouse IgG1κ	WB ICC	0.1 µg/ml 2 µg/ml	Prionics, Switzerland
8H4	anti-prion protein mAb	human a.a. 175-185	mouse IgG1κ	ICC	2 µg/ml	AllPrion, Switzerland
Antibodies						
GFAP	anti-glial fibrillary acidic protein	n/a	rabbit polyclonal	ICC	1.6 µg/ml	Dako, UK
NANOG	anti-human Nanog - stem cells pluripotency	human Nanog - homeobox family	goat IgG	ICC	10 µg/ml	R&D Systems, UK
caveolin 1	caveolae antibody	caveolin 1	rabbit polyclonal	ICC	2 µg/ml	Abcam, UK
clathrin	clathrin heavy chain antibody	clathrin heavy chains	rabbit polyclonal	ICC	1 µg/ml	Abcam, UK
EEA1	early endosome antibody	human EEA1	rabbit polyclonal	ICC	2 µg/ml	Abcam, UK
giantin	golgi antibody	human giantin	rabbit polyclonal	ICC	1 µg/ml	Abcam, UK
calnexin	ER membrane antibody	human calnexin i.e. IP90, p88 & p90	rabbit polyclonal	ICC	20 µg/ml	Abcam, UK
LAMP1	lysosome antibody	human LAMP1 i.e.Igp120, IgpA	rabbit polyclonal	ICC	2.25 µg/ml	Abcam, UK
LAMP2b	lysosome antibody	human LAMP2b	rabbit	ICC	1.25 µg/ml	Abcam, UK
rab11A	protein trafficking antibody	human rab11A of ras superfamily	rabbit polyclonal	ICC	2.5 µg/ml	Abcam, UK
DAPI	nucleic acid stain	AT regions of DNA	n/a	ICC	0.2 µg/ml	Invitrogen, UK
Secondary Ab's						
HRP linked Ab	horseradish peroxidase conjugated Ab	mouse IgG	sheep anti-mouse IgG	WB	0.1 µg/ml	GE Healthcare,
Alexa Fluor 488	fluorescent bright green dye labeled Ab	mouse IgG heavy & light chains	goat anti-mouse IgG	ICC	10 µg/ml	Invitrogen, UK
Alexa Fluor 568	fluorescent bright orange-red dye labeled	goat IgG heavy & light chains	donkey anti-goat IgG	ICC	5 µg/ml	Invitrogen, UK
Alexa Fluor 546	fluorescent bright red dye labeled Ab	rabbit IgG heavy & light chains	donkey anti-rabbit IgG	ICC	10 µg/ml	Invitrogen, UK

Table 2.4: List of antibodies and markers used in the study

2.5 CELL CULTURE GENOTYPING

DNA was extracted from cell culture samples and genetic data obtained by restriction fragment length polymorphism analysis (RFLP) of the prion protein gene *(PRNP)* using restriction enzymes specific to the gene polymorphism under investigation as described previously (Bishop *et al.,* 2009).

2.5.1 Isolation of DNA

DNA from cell cultures was obtained by lysis of cell pellets (approximate volumes of cell pellets was 40 µl) and column purification using the DNA Blood Mini Kit (Qiagen, UK) according to manufacturer's instruction.

2.5.2 PCR-RFLP Analysis

Amplification of the *PRNP* gene sequence (NCBI Accession: AL133396) by the polymerase chain reaction (PCR) involved a forward primer (5'-TGA TAC CAT TGC TAT GCA CTC ATT C-3') and reverse primer (5'-GAC ACC ACC ACT AAA AGG GCT GCA G-3') at 5 pM each per reaction (Eurofins MWG Operon, Germany), that are specific for a 956 bp sequence. Each reaction contained 2 mM $MgCl_2$ (Qiagen, UK), 0.2 mM dNTPs (Promega, UK), and 1 unit of Taq Polymerase – HotStarTaq (Qiagen, UK). The thermal cycling program included an annealing temperature step-down from 65 °C to 60 °C over ten cycles followed by 30 cycles at 60 °C. The PCR product was analysed by 1.5% agarose gel electrophoresis and SYBR green staining.

Confirmation of the codon 129 genotype was performed by restriction enzyme digestion at 37 °C with NspI (New England Biolabs, UK). This enzyme cleaves the

amplicon at *PRNP* codon 155 and at codon 129 only when the latter sequence codes for valine (-GTG-). This allowed for discrimination of the three genotypes: MM, MV, and VV by 1.5% agarose gel electrophoresis and SYBR green staining. Images were captured using ChemiDoc XRS imaging system (BioRad, UK).

2.6 SAMPLE PREPARATION FOR WESTERN BLOT ANALYSIS

2.6.1 Brain sample preparation for Western blot analysis

Detection of PrP^{Sc} in brain homogenates was confirmed by proteinase K digestion and Western blot analysis as described previously (Yull *et al.*, 2006).

In short, wet tissue weighing of 90 – 100 mg was taken from frozen samples. Micro-pestles (Eppendorf, UK) were used to homogenise each sample at 4 °C in extraction buffer (0.5% NP-40, 0.5% sodium deoxycholate, PBS-T pH 7.4, dH_2O) to obtain a 10% weight/volume (w/v) brain homogenate. A non-ionic detergent insoluble pellet (nucleocytoskeletal fraction) was cleared from the supernatant by centrifugation at 2,000 rpm for 5 minutes at 4 °C (Eppendorf 5417R, UK). 100 µl of the detergent soluble supernatant (cytoplasmic and membrane fraction) was digested using proteinase K (PK) (VWR International, UK) at a final concentration of 50 µg/ml at 37 °C for 1 hour. Protease digestion was terminated by the addition of Pefabloc SC (Roche, UK) to a final concentration of 1 mM and the sample was stored at -80 °C or used immediately for analysis.

2.6.2 Harvesting HK cells for Western blot analysis

2.6.2.1 Detecting PrP^C in HK cell culture

Cells at around 90% confluence in a T25 flask were washed twice with 4 °C PBS then lysed for 15 minutes with 100 µl of 4 °C extraction buffer and collected using a cell culture scraper (Fisher Scientific, UK) into safe-lock Eppendorf tubes. An appropriate volume of cell culture suspension (1-5 µl) was mixed with sample buffer, boiled at 100 °C for 10 minutes and subjected to Western blot analysis.

2.6.2.2 Detecting PrP^{Sc} in cultures exposed to brain homogenate

Cells at around 90% confluence were washed twice with 4 °C PBS then lysed for 15 minutes with 100 µl of 4 °C extraction buffer and collected using a cell culture scraper into safe-lock Eppendorf tubes. Then the samples were digested with PK at a concentration 50 µg/ml at 37 °C for 60 minutes. The digestion was terminated by incubating with 1 mM Pefablock SC. A centrifugation step of 14,000 rpm for 60 minutes at 4 ^oC was applied to concentrate the lower levels of PrP^{Sc} in the samples (Lee *et al.*, 2000). The pellets were resuspended in an appropriate volume of sample buffer boiled at 100 °C for 10 minutes and then analysed by Western blotting.

2.6.3 Detection of PrP^{Sc} in culture medium and cell PBS washes

Brain spiked medium (or control medium) incubated with cell cultures for the desired time period was aspirated and transferred into safe-lock Eppendorf tubes. Then the cells were rinsed with 1 ml of PBS (4X) which was then also transferred to individual safe-lock Eppendorf tubes. The samples were PK digested (concentration 50 µg/ml) at 37 °C for 60 minutes. The digestion was terminated by incubation with 1 mM Pefablock SC, pellets were collected by centrifugation at 14,000 rpm for 60

minutes. The supernatant was aspirated and disposed of. Pellets were resuspended in an appropriate volume of sample buffer, boiled at 100 °C for 10 minutes and analysed by Western blotting.

2.7 WESTERN BLOT ANALYSIS

An equal volume of 4X NuPAGE LDS sample buffer (Invitrogen, UK) was added to each sample aliquot to a final concentration 1X and boiled at 100 °C for 10 minutes. Samples were briefly collected by centrifugation and loaded onto a NuPAGE Novex 10% Bis-Tris gel (Invitrogen, UK) and subjected to electrophoresis for 50 minutes at 200 volts using pre-set gel cassettes (Invitrogen, UK) and NuPAGE MES-SDS 1X running buffer (Invitrogen, UK). A benchmark (Pre-stained Protein Ladder, Invitrogen, UK) and a Magic Marker (Western Protein Standard, Invitrogen, UK) were run alongside the samples. The former is visible during electrophoresis and on the transfer, the latter is detected by the secondary antibody on the blot.

The gels were then electroblotted onto polyvinylidene diflouride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotechnology, UK) for 1 hour at 30 volts. Prior to electrobloting the PVDF membrane was wet in MeOH (Fisher Scientific, UK), rinsed with dH_2O and equilibrated for 10 minutes in 1X Transfer buffer consisted of 4% 20X NuPAGE® Transfer Buffer (Invitrogen, UK), 16% MeOH, 80% dH_2O. Gel was sandwiched into a transfer cassette in the following manner: 3 sponges pre-soaked in transfer buffer/2 pre-soaked blotting papers/gel/PVDF membrane/2 pre-soaked blotting papers/3 pre-soaked sponges.

For immunodetection, the PVDF membrane was blocked with solution of 5% (w/v) non-fat milk powder (Sigma-Aldrich, UK) dissolved in TBS-T (2 mM Tris/HCl, pH

7.6, 150 mM NaCl, containing 0.1% Tween 20) (Sigma-Aldrich, UK) for 60 minutes. Then the PVDF membrane was washed three times with TBS-T and incubated with anti-PrP monoclonal antibody 3F4 or 6H4 diluted in TBS-T for 60 minutes. The PVDF membrane was washed three times (3 minutes each wash) with TBS-T and incubate with horseradish peroxidise conjugated sheep anti-mouse IgG antibody diluted in TBS-T for 60 minutes. Following four washes (5 minutes each) in TBS-T, the membranes were developed using ECL Plus (Amersham Pharmacia Biotechnology, UK) and imaged on Hyperfilm ECL X-ray film (Amersham Pharmacia Biotechnology, UK) using a Konica-Minolta Hyperprocessor. X-ray film images were captured using a Bio-Rad GS-800 scanning densitometer.

2.8 PROTEIN-MISFOLDING CYCLIC AMPLIFICATION (PMCA)

2.8.1 Preparation of substrate

Preparation of the substrates for PMCA is the most critical step in achieving successful amplification. HK cell homogenates were prepared as a substrate for PMCA as follows: confluent cell cultures in T25 flasks were washed twice with 4 °C sterile PBS and scraped in 1 ml of PBS using cell scraper. The cell suspension was collected in a 50 ml Falcon tube (Corning, UK) and centrifuged at 2,400 rpm for 15 minutes at 4 °C using rotor 11180 in Sigma 3-16K centrifuge. The supernatant was aspirated and the cell pellet was homogenised (10% w/v) at 4 °C in PMCA conversion buffer (PBS, 0.15 M NaCl, 1% Triton X-100, EDTA, 1X Complete™ protease inhibitor cocktail-Roche, UK) by vortexing and used straight away as a substrate for PMCA. The Complete™ Mini EDTA-free protease inhibitor is a mixture of several protease inhibitors and was used for inhibition of proteolytic activity where EDTA may interfere with protein stability, specially inhibition of

serine, cysteine, but not metalloproteases at a concentration 1 tablet/10 ml PMCA conversion buffer.

Humanised MM transgenic (Hu MM Tg) sagittally bisected frozen half brain tissue (stored at -80 °C) was homogenised (10% w/v) using an Eppendorf micro-pestle in 4 °C PMCA conversion buffer. The homogenate was cleared of particulate matter by centrifugation at 2,000 rpm for 10 seconds at 4 °C and the collected supernatant was used straight away as a substrate for PMCA.

2.8.2 Preparation of brain extracts for PMCA experiment (seeds)

Human brain (temporal cortex) or animal brain tissues were homogenised in 4 °C PMCA conversion buffer at 10% w/v using eppendorf micro-pestles. The homogenates were cleared of particulate matter by centrifugation at 4,300 rpm for 5 minutes at 4 °C, supernatants were divided into aliquots and retained for further use in PMCA experiment at -80 °C. An aliquote of the sample was PK digested and analysed by Western blot as described in section 2.6.1 and 2.7

2.8.3 PMCA reaction

Design of PMCA reactions was as follows: substrate was seeded with a dilution of brain homogenate (previously titrated by Western blot) such that the seed material contained sufficient PrP^{res} to be detectable at the lower end of the linear range of a standard Western blot. Reactions were prepared in duplicate. The negative control (unamplified aliquot) was immediately frozen at -80 °C and the PMCA aliquot was subjected to the PMCA reaction in a sealed 96 well PCR plate (Sarsted, UK). The experiment reaction consists from 48 cycles of 40 seconds sonication at 80% total

power output and 29 minutes 20 seconds incubation at 37 °C for 24 hours using a Sonicator (Model 3000, Misonix, USA).

2.8.4 Analysis of PMCA product by western blotting

SDS was added at a final concentration of 0.04% to thawed non-PMCA control reaction and to the PMCA reaction products. This was followed by PK digestion of samples at a concentration 50 μg/ml at 37 °C for 60 minutes. The digestion reaction was terminated by addition of 1 mM Pefabloc SC. Samples were mixed with an appropriate volume of 4X NuPAGE LDS sample buffer and boiled at 100 °C for 10 minutes before Western blot analysis.

2.9 INFECTION STUDIES OF HK CELLS

2.9.1 Preparation of brain homogenates for cell exposure studies

Brain tissues were homogenised at 10% (w/v) in a sterile 4 °C PBS/5% glucose solution using Eppendorf micro-pestles. The homogenates were cleared of particulate matter by centrifugation at 2000 rpm for 5 minutes at 4 °C. Supernatants were divided into aliquots and stored at -20 °C for further use. One aliquot of each brain homogenate was retained, PK digested and analysed by Western blotting for the detection and typing of PrP^{Sc} in the sample.

HK cells were challenged with infectious brain homogenates in many sets of experiments, using different methodologies previously described to result in successful infections *in vitro* (Nishida *et al.*, 2000; Sabuncu *et al.*, 2003; Cronier *et al.*, 2004; Lehmann and Milhavet, 2004; Weissmann and Aguzzi, 2005; Milhavet *et al.*, 2006; Dlakic *et al.*, 2007; Courageot *et al.*, 2008). Cells were incubated with

brain homogenates and then tested for PrP^{Sc} production after each passage or at stated time points.

2.9.2 Attempts at infection of HK cells

General method

HK cells were routinely cultured in T25 flasks and after reaching 50-60% confluence, the cultures were exposed to brain spiked media prepared according to one of following descriptions (or see Tables 2.5-11 in this section - 2.9.2). The method by which cells were harvested and pre-treated for detection of PrP^{Sc} by Western blot is described in section 2.6.2.2.

Methodological variations

2.9.2.1 First set

♦ In the first set of experiments the HK cells were challenged with 0.1% dilution of disease brain homogenates (Table 2.5: $1._1$–$1._8$) according to the published methods (Sabuncu *et al.*, 2003; Cronier *et al.*, 2004; Milhavet *et al.*, 2006; Dlakic *et al.*, 2007). Cells were exposed to medium containing brain homogenate for 24 hours. To avoid contaminations by conventional agents partial decontamination of the homogenates with two alternative methods was attempted (Lehmann and Milhavet, 2004; Milhavet *et al.*, 2006):

a). The 10% brain homogenate was warmed for 20 minutes at 60 °C in a heat-block and the clumps of brain tissue resulting from the heating were resuspended and broken up.

b). Medium containing 0.1% brain homogenate was filtered through a 0.22 µm sterile filter (Millex®GV, UK).

A few flasks of HK cells were also incubated with 0.1% dilution of the brain homogenate in the medium without any pre-treatment.

Cells were harvested after 16 days of culturing for Western blot analysis using the 6H4 antibody (the medium had been changed five times and cells had been passaged once).

No.	INOCULUM	TREATING OF INOCULUM			INCUBATION TIME	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical				
		Filte-red	Heat treated	Non-sterilised		
1.$_1$	0.1% iCJD$^{GHT}{}_1$	+			24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_2$	0.1% iCJD$^{GHT}{}_1$		+		24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_3$	0.1% iCJD$^{GHT}{}_1$			+	24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_4$	0.1% vCJD	+			24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_5$	0.1% vCJD			+	24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_6$	0.1% BSE_1	+			24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_7$	0.1% BSE_1			+	24 hours	16 days: 1 passage, 5 med. changes (1x time point)
1.$_8$	0.1% AD			+	24 hours	16 days: 1 passage, 5 med. changes (1x time point)

Table 2.5: Conditions used in the first set of HK cell challenges

2.9.2.2 Second set

♦ The second set of HK cell challenges (Table 2.6: 2.$_1$-2.$_{16}$) tested three different concentrations of infectious brain homogenates – 0.1%, 0.5% and 1% treated with 1X extraction buffer (0.5% NP-40, 0.5% sodium deoxycholate, PBS-T pH 7.4, dH_2O) or 1X Triton-DOC lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl – pH 7.5, 2 mM EDTA + protease inhibitors) according to published methods (Nishida *et al*., 2000; Milhavet *et al*., 2006). Cells were also exposed to untreated brain homogenates. The incubation time in this experiment was 24 hours. In this set, freshly passaged cells were also exposed to infectious brain homogenates – a pellet of HK cells was gently resuspended in brain

spiked medium and cells in this suspension were then plated into fresh culture flask. Cells were harvested for Western blot analysis after 26 days of culturing (medium had been changed 10 times and cells were passaged once).

No.	INOCULUM	TREATING OF INOCULUM				INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical		Chemical			
		Filte-red	Non-sterilised	Extraction buffer	Triton X-100		
2.$_1$	0.1% iCJD$^{GHT}{}_1$	+		+		24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_2$	0.1% iCJD$^{GHT}{}_1$		+	+		24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_3$	0.1% iCJD$^{GHT}{}_1$		+	+		24 hours *	26 days:1 passage, 10 med. changes (1x time point)
2.$_4$	0.1% iCJD$^{GHT}{}_1$	+			+	24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_5$	0.1% iCJD$^{GHT}{}_1$	+			+	24 hours *	26 days:1 passage, 10 med. changes (1x time point)
2.$_6$	0.1% iCJD$^{GHT}{}_1$		+		+	24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_7$	0.1% iCJD$^{GHT}{}_1$		+		+	24 hours *	26 days:1 passage, 10 med. changes (1x time point)
2.$_8$	0.1% AD		+			24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_9$	0.5% iCJD$^{GHT}{}_1$		+			24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{10}$	0.5% AD		+			24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{11}$	1% iCJD$^{GHT}{}_1$	+		+		24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{12}$	1% iCJD$^{GHT}{}_1$		+	+		24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{13}$	1% iCJD$^{GHT}{}_1$	+			+	24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{14}$	1% iCJD$^{GHT}{}_1$		+		+	24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{15}$	1% AD	+			+	24 hours	26 days:1 passage, 10 med. changes (1x time point)
2.$_{16}$	1% AD		+		+	24 hours	26 days:1 passage, 10 med. changes (1x time point)

Table 2.6: Conditions used in the second set of HK cell challenges
*- freshly passaged cells

2.9.2.3 Third set

♦ The third set of HK challenges (Table 2.7: 3.$_1$-3.$_7$) included modifications in the treatment of the brain homogenates prior to incubation with the HK cells. According to successful infection using the method of Courageot *et al.*, (2008) the 10% w/v disease brain homogenates were homogenised in a Ryboliser (FastPrep FP120, Germany) for 45 seconds at speed 5.5 and then subsequently sonicated by a Sonicator (Model 3000, Misonix, USA) at total power output ~ 300 watts for 1 minute. These treated brain homogenates were diluted into culture media in three different concentrations: 0.1%, 0.5% and 2%. The inoculum was removed after 24 or 68 hours of incubation with the cells and cells were harvested for Western blot

analysis after 22 days of culturing (the medium had been changed eight times and cells were passaged once).

No.	INOCULUM	TREATING OF INOCULUM			INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical				
		Soni-cated	Rybo-lised	Non-sterilised		
$3._1$	0.1% $iCJD^{GHT}{}_1$	+	+	+	68 hours	22 days:1 passage, 8 med. changes (1x time point)
$3._2$	0.1% $iCJD^{GHT}{}_1$	+	+	+	68 hours *	22 days:1 passage, 8 med. changes (1x time point)
$3._3$	0.1% AD	+	+	+	68 hours	22 days:1 passage, 8 med. changes (1x time point)
$3._4$	0.5% $iCJD^{GHT}{}_1$	+	+	+	68 hours	22 days:1 passage, 8 med. changes (1x time point)
$3._5$	0.5% $iCJD^{GHT}{}_1$	+	+	+	24 hours *	22 days:1 passage, 8 med. changes (1x time point)
$3._6$	2% $iCJD^{GHT}{}_1$	+	+	+	24 hours	22 days:1 passage, 8 med. changes (1x time point)
$3._7$	2% AD	+	+	+	24 hours	22 days:1 passage, 8 med. changes (1x time point)

Table 2.7: Conditions used in the third set of HK cell challenges
*- freshly passaged cells

2.9.2.4 Fourth set

♦ In the fourth set of experiments the HK cells were challenged (Table 2.8: $4._1$-$4._{14}$) with ribolysed and sonicated disease brain homogenates at five different concentrations (2%, 1.5%, 1%, 0.5% and 0.1%). After 24 hours, the inoculum was removed, cells were washed and fresh (brain-free) culture medium was added. The challenged cells were then cultivated for four days, then split 1:2 and cultured for additional four days when they were harvested for Western blot analysis. The experiment was carried out in duplicate. Approximately every five days one flask from each type of the challenge was used for subcultivation, whilst the other was used to prepare a cell lysate for Western blot analysis (see Figure 2.1). The cells were cultured for 42 days in total (medium had been changed 15 times and cells passaged eight times).

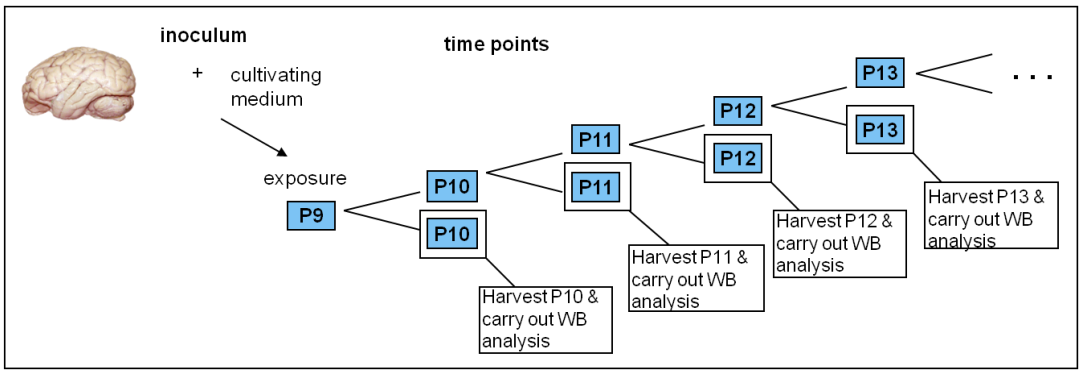


Figure 2.1: Methodology used for detecting any *de novo* formed PrP^{Sc} by HK cells exposed to infectious brain homogenate

No.	INOCULUM	TREATING OF INOCULUM				INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical			Che-mical		
		Soni-cated	Rybo-lised	Non-sterilised	Triton X-100		
4.$_1$	0.1% iCJD$^{GHT}{}_1$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_2$	0.1% iCJD$^{GHT}{}_1$			+	+	24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_3$	0.5% iCJD$^{GHT}{}_1$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_4$	0.5% iCJD$^{GHT}{}_1$	+	+	+		24 hours *	42 days:8 passages, 15 med. changes (8x time point)
4.$_5$	0.5% iCJD$^{GHT}{}_2$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_6$	0.5% iCJD$^{GHT}{}_2$	+	+	+		24 hours *	42 days:8 passages, 15 med. changes (8x time point)
4.$_7$	1% iCJD$^{GHT}{}_1$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_8$	1.5% iCJD$^{GHT}{}_1$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_9$	2% iCJD$^{GHT}{}_1$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_{10}$	2% iCJD$^{GHT}{}_2$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_{11}$	2% iCJD$^{GHT}{}_3$	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_{12}$	2% vCJD	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_{13}$	2% BSE_2	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)
4.$_{14}$	2% AD	+	+	+		24 hours	42 days:8 passages, 15 med. changes (8x time point)

Table 2.8: Conditions used in the fourth set of HK cell challenges
*- freshly passaged cells

2.9.2.5 Fifth set

♦ The fifth set of experiments the HK cell challenges (Table 2.9: 5.$_1$-5.$_{13}$) included using an immobilised infectious brain homogenate. The 10% w/v brain homogenates were either treated with minute amount of Triton X-100, or rybolised and subsequently diluted in PBS. The brain dilutions (100 µl of either a 1% or 0.5 % homogenate) were "painted" on the bottom of the T25 flasks and dried overnight in antiseptic conditions. The next day, the flasks were washed once with PBS and HK

cells were plated onto the base thus prepared. Cells were incubated with inoculum for four days, then rinsed with PBS, supplied with fresh culture medium and further maintained for three days before first harvesting for Western blot analysis. The experiment was carried out in duplicate. Cells were harvested for PrP^{Sc} analysis by Western blot six times and passaged (1:2) five times over the 26 days of culturing.

No.	INOCULUM	TREATING OF INOCULUM				INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical		Chemical	Painted on the bottom of the flask		
		Rybo-lised	Non-sterilised	Triton X-100			
5.$_1$	0.5% iCJD$^{GHT}{}_1$		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_2$	1% iCJD$^{GHT}{}_1$		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_3$	0.5% iCJD$^{GHT}{}_2$		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_4$	0.5% vCJD		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_5$	0.5% BSE_2		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_6$	0.5% AD		+	+	+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_7$	0.5% iCJD$^{GHT}{}_1$	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_8$	1% iCJD$^{GHT}{}_1$	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_9$	0.5% iCJD$^{GHT}{}_2$	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_{10}$	0.5% iCJD$^{GHT}{}_3$	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_{11}$	0.5% vCJD	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_{12}$	0.5% BSE_2	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)
5.$_{13}$	0.5% AD	+	+		+	4 days	26 days:5 passages, 9 med. changes (6x time point)

Table 2.9: Conditions used in the fifth set of HK cell challenges

2.9.2.6 Sixth set

♦ In sixth set of experiments (Table 2.10: 6.$_1$) the pellet of HK cells was gently resuspended in ribolysed and sonicated 0.5% $iCJD_2$ brain spiked medium with omitted Penicillin/Streptomycin/Amphotericin antibiotics/antimycotics and cells were plated into fresh cell culture flasks. This strategy was inspired by preliminary results from a cell culture studies (Mange *et al.*, 2000a; Mange *et al.*, 2000b; Mange and Lehmann, 2002; Weissmann and Aguzzi, 2005; Soler *et al.*, 2008). Freshly passaged cells were incubated with 2 ml of this inoculum for four days (3 days, then 2 ml of fresh, no antibiotics containing, medium was added and cells were cultured for one additional day). Then the cells were washed, given fresh, no antibiotics

containing, medium and cultured for another 4 days before first harvesting for Western blot analysis. The experiment was carried out in duplicate. One flask of cells was harvested for Western blot analysis and the other flask was split into two flasks and cultured until confluent when harvesting and splitting process was repeated. Cells were passaged four times and harvested for Western blot analysis at five time points in total (29 days, passage every ~ 5 days).

No.	INOCULUM	TREATING OF INOCULUM			INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical				
		Soni-cated	Rybo-lised	Non-sterilised		
6.$_1$	0.5% iCJD$^{GHT}{}_2$	+	+	+	4 days* No ATB	29 days:4 passages, 14 med. changes (5x time point)

Table 2.10: Conditions used in the sixth set of HK cell challenges
*- freshly passaged cells

2.9.2.7 Seventh set

♦ The seventh set of experiments (Table 2.11: 7.$_1$-7.$_2$) investigated the potential of increasing the cell susceptibility to infection with prions after being cultured in nutrient-low conditions. HK cells were either maintained in normal medium (containing 10% Foetal Calf Serum, FCS) or nutrition-low medium (containing 0.5% FCS) for 3 days prior exposure to growth hormone associated sonicated 1% iCJD$_1$ brain spiked medium. The brain homogenate was diluted in a medium either containing 10% or 0.5% FCS, according to the type of medium cells were cultured prior to exposure. The experiment was carried out in duplicate. Cells were incubated with the brain spiked medium for 48 hours. Cells were harvested for Western blot analysis using 3F4 primary antibody at 24 and 48 hours time period of continuous exposure. The brain spiked medium was then removed, cells were rinsed with PBS and further maintained with fresh culture medium (containing either 10% or 0.5% FCS) for 3 days. At this time point (3rd day of recovery) the challenged HK cells

were also harvested for Western blot analysis. In the next medium change, all the challenged cells were given complete culture medium (containing 10% FCS). Medium changes took places every three days. Analysis for PrP^{Sc} was then carried out on 6^{th}, 9^{th} day of culturing with complete (brain-free) medium, also after first passage on 12^{th}, 24^{th} and second passage on 40^{th} day of recovery. Cells were passaged two times and harvested for Western blot analysis at eight time points in total (42 days, passage every 20 days).

In this assay the medium cultured with the cells was also retained and analysed for PrP^{Sc} by Western blot (as described in section 2.6.3).

No.	INOCULUM	TREATING OF INOCULUM		CULTURE MEDIA CONTAINING	INCUBATION WITH INFECTIOUS MEDIUM	CHALLENGED CELLS HARVESTED FOR WB ANALYSIS AFTER
		Physical				
		Soni-cated	Non-sterilised			
7.$_1$	1% iCJD$^{GHT}{}_1$	+	+	10% FCS	48 hours	42 days:2 passages, 13 med. changes (8x time point)
7.$_2$	1% iCJD$^{GHT}{}_1$	+	+	0.5% FCS	48 hours	42 days:2 passages, 13 med. changes (8x time point)

Table 2.11: Conditions used in the seventh set of HK cell challenges

2.10 PRP^{SC} UPTAKE BY HK CELLS ANALYSED BY WESTERN BLOT

HK cells were challenged with dilutions of crude sonicated extract of iCJD or vCJD brain homogenates (preparation described in section 2.11.1.1). The exposed cells were harvested (method described in section 2.6.2.2) at early and late time points during continuous exposure and analysed by Western Blot. In addition to the exposed cells, the brain spiked medium (incubated with the cells for defined time periods), the 1^{st} PBS wash and 4^{th} PBS wash were collected (section 2.6.3) and analysed by Western blot. Four different experimental designs were used (section 2.10.1-4).

2.10.1 Scheme of HK cell culture analysis after incubation with brain spiked medium (time points: 30 minutes, 1, 2, 4, 6, 24 and 48 hours)

HK cells were exposed to a complete cell culture medium containing prion disease brain homogenate ($iCJD_1$ or vCJD). The cells were analysed for cell associated PrP^{Sc} at various time points after continuous incubation with brain-spiked medium (30 minutes, 1, 2, 4, 6, 24, and 48 hours) (Figure 2.2). Samples analysed at each time point were: exposed cells (extensively washed), medium (incubated with cells), 1st and 4th PBS wash (carried out prior to cell harvesting). Samples were treated with PK for 60 minutes at 37 °C at a concentration 50 µg/ml, then pelleted by centrifugation at 14,000 rpm for 60 minutes and subjected to Western blot analysis with anti-PrP mAb 3F4.

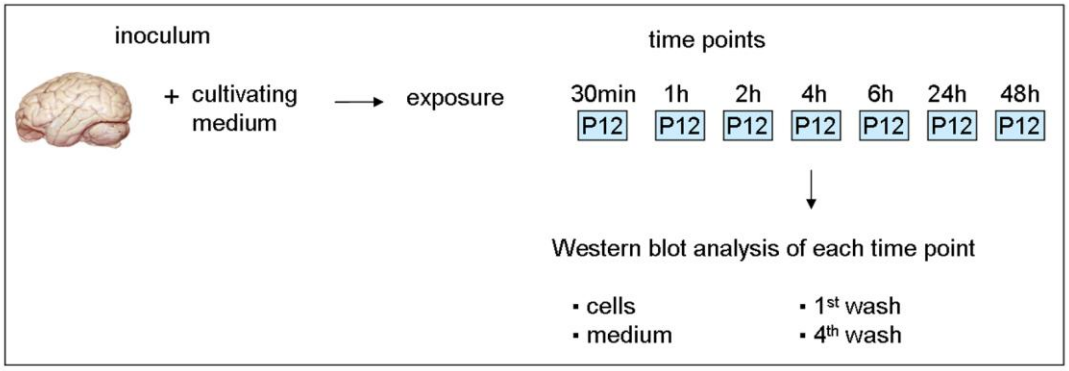


Figure 2.2: Scheme of HK cell culture analysis after incubation with brain spiked medium (time points: 30 minutes - 48 hours)

2.10.2 Scheme of HK cell culture analysis after incubation with brain spiked medium (time points: 1, 3, 6, 24, 48 hours of continuous exposure, 1st, 2nd and 3rd cell passage post exposure – 51 days)

Pelleted HK cells were gently resuspended in complete cell culture medium containing $iCJD_1$ brain homogenate. Cells were plated into fresh cell culture flasks and continuously incubated with the infectious medium until harvested for analysis of cell associated PrP^{Sc} at specific time points (1, 3, 6, 24, and 48 hours). After

48 hours of continuous exposure, the cells were extensively washed, split at a ratio of 1:2 and allowed to grow until confluent. At this point one flask of cells was harvested as another time point for Western blot analysis and the other flask was again split into two further flasks. Cells were again allowed to grow until confluent when harvesting and splitting process was repeated. Samples analysed from each time points (Figure 2.3) were: exposed cells (extensively washed), medium (incubated with cells), 1st and 4th PBS wash (carried out prior cell harvesting). Samples were treated with PK for 60 minutes at 37 °C at a concentration 50 µg/ml. Then insoluble proteins were pelleted by centrifugation at 14,000 rpm for 60 minutes and subjected to Western blot analysis with anti-PrP mAb 3F4.

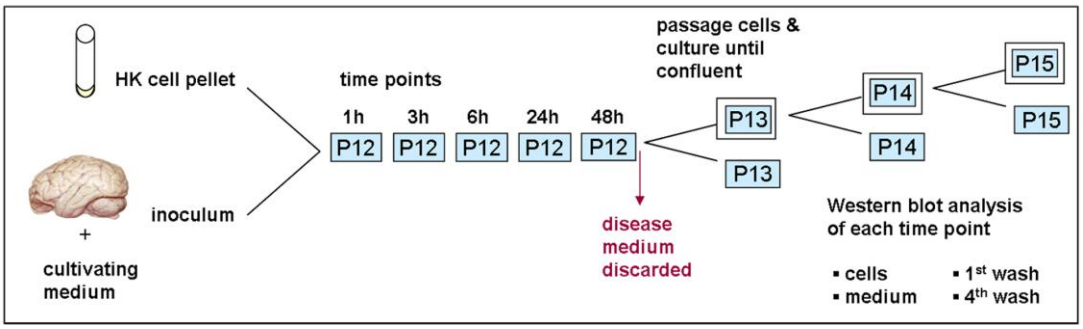


Figure 2.3: Scheme of HK cell culture analysis after 48 h continuous incubation with iCJD brain spiked medium followed by culturing of the cells for 51 days

2.10.3 Scheme of HK cell culture analysis after incubation with brain spiked medium (time points: 1, 3, 6, 24 hours of continuous exposure followed by a 24 hours recovery and culturing of the cells until senescent – 1st to 7th passage – 177 days)

HK cells were continuously exposed to the complete cell culture medium spiked with $iCJD_1$ brain homogenate and analysed for cell associated PrP^{Sc} at 1, 3, 6 and 24 hours time points. At the 24 hour time point the cells were extensively washed and allowed to grow in fresh (brain homogenate free) medium for 24 hours – recovery (48 hours

time point). Then the cells were split at ratio 1:2 and cultured until confluent. One flask of cells was harvested for Western blot analysis, the other flask was again split into two flasks and the cells were allowed to grow until confluent when harvesting and splitting process was repeated. This process was repeated until passage 19 (p19, 177 days post exposure to brain homogenate) (Figure 2.4) when cells became senescent and stopped dividing. At this point the experiment was terminated. Samples analysed from each time point: exposed cells (extensively washed), medium (incubated with cells), 1st and 4th PBS wash (carried out prior cell harvesting). Samples were treated with PK for 60 minutes at 37 °C at a concentration 50 µg/ml, then insoluble proteins were pelleted by centrifugation at 14,000 rpm for 60 minutes and subjected to Western blot analysis with anti-PrP mAb 3F4.

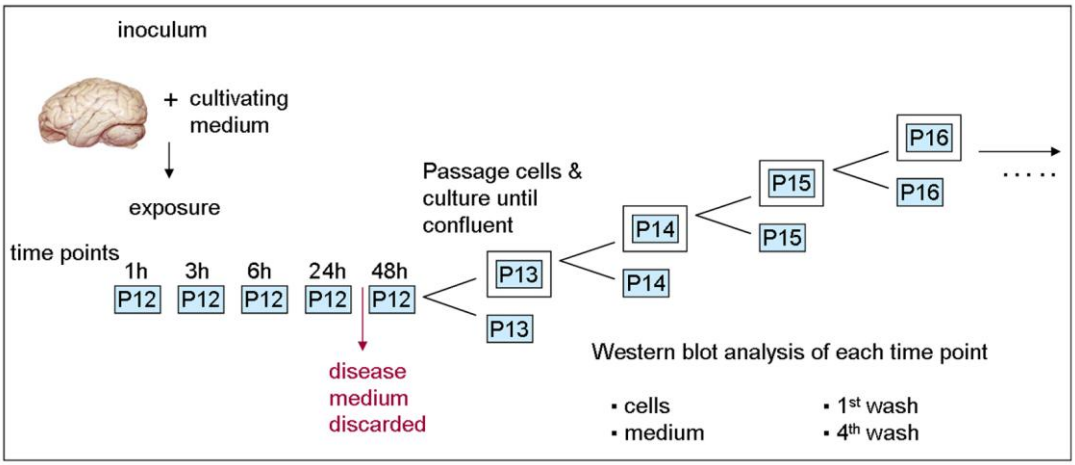


Figure 2.4: Scheme of analysis of cell associated PrP^{Sc} after HK cells continuous incubation with iCJD brain homogenate for 24 hours followed by a 24 hour recovery and culturing of the cells until senescent

2.10.4 Scheme of HK cell culture analysis after incubation with brain spiked medium (time points: 1, 5, 24, 48 hours of continuous exposure followed by a 1, 3, 24, 48, 120 hours recovery and 1st passage)

HK cells were continuously exposed to complete cell culture medium containing $iCJD_1$ brain homogenate up to 48 hours and analysed for cell associated PrP^{Sc} at 1, 5, 24, and 48 hours of the exposure time. The cells were then extensively washed and allowed to grow in fresh complete medium up to 120 hours with three medium changes - recovery. Cells were analysed for cell associated PrP^{Sc} at 1, 3, 24, 48, and 120 hours of the recovery time. Subsequently, the cells were split at ratio 1:2 and grown until confluent when analysed as another time point (1st passage) (Figure 2.5). Samples analysed from each time point were: exposed cells (extensively washed), medium (incubated with cells), 1st and 4th PBS wash (carried out prior cell harvesting). Samples were treated with PK for 60 minutes at 37 °C at a concentration 50 μg/ml, then insoluble proteins were pelleted by centrifugation at 14,000 rpm for 60 minutes and subjected to Western blot analysis with anti-PrP mAb 6H4.

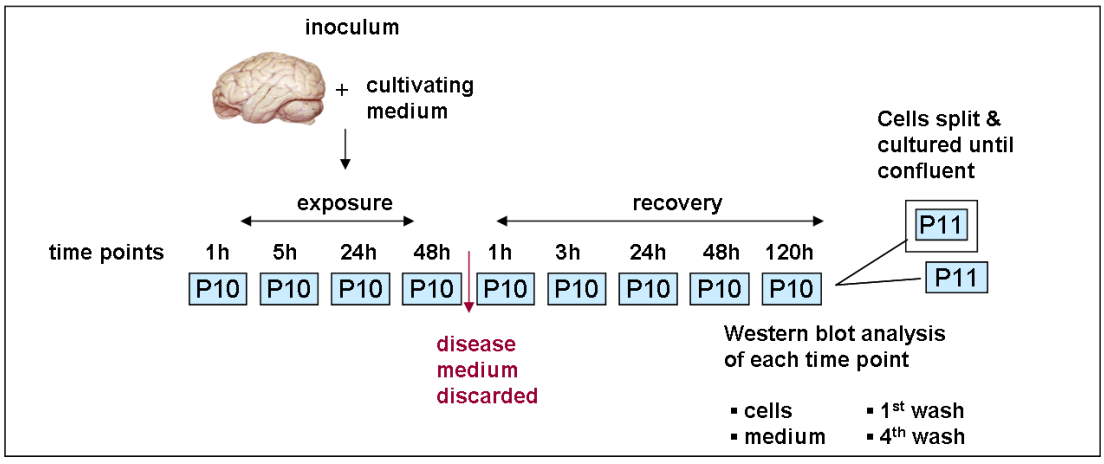


Figure 2.5: Scheme of analysis of cell associated PrP^{Sc} after HK cells continuous incubation with iCJD brain homogenate for 48 hours followed by a 120 hour recovery and one cell passage

2.10.5 Densitometric evaluation of PrP^{Sc} uptake by HK cells analysed by Western blot

The uptake of PrP^{Sc} by HK cells exposed to medium containing prion diseased brain homogenates was monitored by Western blot analysis and the results were evaluated by densitometry. This was performed to provide a quantitative assessment of any increase of the cell associated PrP^{Sc} with time of cell exposure to prion spiked medium. However, it is important to note the limitations of this approach and that Western blotting is well recognised to be a semi-quantitative method at best. The dynamic range of Western blotting (as performed in this thesis) is circumscribed by the use of X-ray film, which has a linear range of less than 1 log (as can be determined empirically). In practice the main constraint tends to be that of saturation at high signal, when all local silver grains have been activated and further activation only results from lateral spread and increased size of a band rather than increased density. Because PrP^{res} typically has a three band pattern, each with different abundance, the linear range of response is further constrained when all three bands are being measured. The background signal on individual blots is also an important consideration since this can vary and must be subtracted before data is compared. Individual identical and non-identical experiments were therefore analysed seperately. For these reasons the data can only legitimately be viewed in support of a trend rather than as a quantitative measure.

The analysis was carried out as follows: the images from the X-ray films were scanned using a ChemiDoc™ XRS+ System (Bio-Rad) with Image Lab™ 2.0 software and the manufacturer's instructions. The individual bands were then bracketed using the volume tool of the software and analysed. A background value for each blot was then determined and subtracted from the samples on the individual

blots. The volume intensity is the pixel value of each investigated band. The background subtracted values were then plotted on a graph using Microsoft Office Excel®.

2.11 PrP^{Sc} UPTAKE STUDIES ANALYSED BY IMMUNOCYTOCHEMISTRY

2.11.1 Preparation of brain spiked medium

2.11.1.1 Cell culture medium spiked with sonicated brain homogenates

Brain tissues were homogenised (10% w/v) in 4 °C PBS/5% glucose (Fisher Scientific, UK) using Eppendorf micro-pestles. The homogenates were cleared of particulate matter by centrifugation at 2,000 rpm for 5 minutes at 4 °C. Then they were disrupted by sonication at total power output ~ 300 watts for 1 minute, and diluted into complete cell culture medium.

2.11.1.2 Cell culture medium spiked with rybolised, sonicated and filtered brain homogenates

Brain tissues were homogenised (10% w/v) in 4 °C PBS/5% glucose using Eppendorf micro-pestles and then ribolysed for 45 seconds at speed 5.5. The homogenates were cleared of particulate matter by centrifugation and sonicated as described in section 2.11.1.1. The resultant homogenate was filtered by spin filters - pore size 220 nm, (Agilent Technoligies, UK) or pore size 450 nm, (Thermo Fisher Scientific, UK) at 10,000 rpm for 5 minutes at 4 °C.

2.11.2 Cell culture exposure to brain spiked medium analysed by immunocytochemistry

2.11.2.1 Continuous exposure studies

Routinely, cells were exposed continuously to brain homogenate at a final w/v concentration of 1% in complete culture medium for desired time periods up to 72 hours, followed by immunocytochemistry for prion protein (and other proteins) and analysed by confocal microscopy.

2.11.2.2 Continuous exposure and recovery studies

In the case of recovery studies the brain spiked medium was withdrawn (after desired continuous exposure), cells were extensively washed with 1X HBSS and allowed to continue growing in the complete cell culture medium (control medium), which was changed every 24 h. Cells were immunostained at desired recovery time points and also passaged, and further cultured until the culture reached 70% confluence, and then immunostained for PrP.

2.11.2.3 PrP^{Sc} "pulse-chase" studies

The method is illustrated in Figure 2.6. Cells were exposed to a medium containing 6H4 (1 µg/ml), either control (unspiked) or brain spiked (sonicated 1% brain homogenate), for 30 minutes at 4 °C and additional 15 minutes at 37 °C, to initiate the internalisation process – "pulse" (A). Then the cells were extensively washed to remove any material deposited on the cells surface and further cultured in control medium for desired time period – "chase" (B). The cells were then immunostained at a different time point of the "chasing" phase. It should be noted that the incubation with anti-prion primary antibody is no longer needed in the immunostaining protocol. The fixed and permeabilised cells were blocked and then incubated with the FITC secondary antibody Alexa 488 labelling the uptaken PrP-6H4 complexes.

Another important fact is that the immunocytochemical procedure often included pre-treatment of the cells with guanidine to diminish PrP^{C} and accentuate PrP^{Sc} signal (D). Subsequently, the samples were washed and immunostained with cell organelle antibodies overnight, which were revealed by incubation with Alexa 546 for 60 minutes. The nuclei were counterstained with DAPI and the slides were then mounted with mounting media and examined by confocal microscopy.

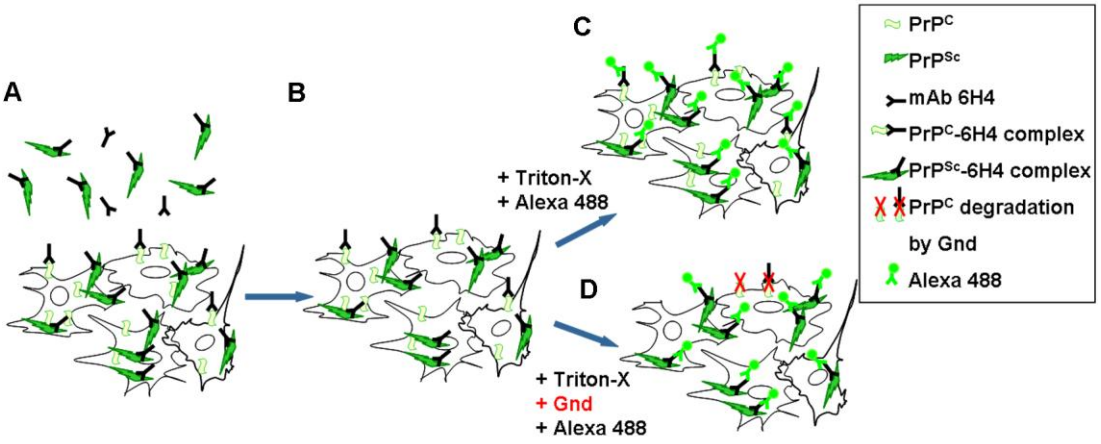


Figure 2.6: Diagrammatic representation of the "pulse and chase" experiment
Cells were incubated with either control or brain spiked medium containing the anti-PrP primary antibody 6H4 (1 µg/ml) for 30 minutes at 4 °C to create an antigen/antibody complex. Then the cells were placed at 37 °C for additional 15 minutes to initiate the internalisation of the antigen/antibody complex – "pulse" (A). The cells were then extensively washed with 1X HBSS solution, fresh control medium was added to cultures and cells were subsequently cultured at 37 °C for desired time period – "chase" (B). Then the medium was aspirated, cells were extensively washed and immunolabeled as described in section 2.12.5. In short, cells were fixed, permeabilised, blocked and either pre-treated with guanidine - enhancing the PrP^{Sc} signal (D) or not treated (C), followed by incubation with Alexa 488 for 60 minutes. Subsequently the samples were washed and immunostained with cell organelle antibodies overnight, which were revealed by incubation with Alexa 546 for 60 minutes. The nuclei were counterstained with DAPI, slides were then mounted with mounting media and examined by confocal microscopy.

2.12 IMMUNOCYTOCHEMISTRY (ICC)

2.12.1 Immunocytochemistry of live cells

Cell culture samples for live cell staining were washed with PBS, blocked with blocking solution 3% bovine serum albumin (BSA, Invitrogen, UK) in Dulbecco's PBS (D'PBS, Invitrogen, UK), and incubated with the primary antibody 6H4 for

60 minutes at RT with shaking at 60 rpm in an orbital shaker. The cells were washed and subsequently incubated with the Alexa 488-conjugated secondary antibody for 60 minutes in blocking solution, followed by counterstaining with DAPI (Invitrogen, UK) for 15 minutes. The slides were mounted with Vectashield mounting media (Vector Laboratories LTD, UK) and examined by confocal microscopy.

2.12.2 Immunocytochemistry of fixed and permeabilised cells

Cell cultures intended to be immunostained fixed and permeabilised were washed with PBS (Invitrogen, UK), fixed in chamber slides for 10 minutes with 4% paraformaldehyde (PFA, GPR, UK) in PBS and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich, UK) in PBS. The cells were then washed and blocked with blocking solution 3% BSA in D'PBS, incubated with the PrP primary antibody in blocking solution for 30 minutes at 37 ^{o}C and then 30 minutes at RT with shaking at 60 rpm in an orbital shaker. The cells were subsequently incubated with the Alexa 488-conjugated secondary antibody for 60 minutes followed by counterstaining with DAPI for 15 minutes. The slides were mounted with Vectashield mounting media and examined by confocal microscopy.

2.12.3 Immunocytochemistry of fixed, permeabilised and denaturated cells

Cells were washed with PBS, fixed with 4% PFA in PBS and permeabilised with 0.1% Triton X-100 in PBS. Protein denaturation was performed by incubation with PK at a concentration 0.3 µg/ml for 9 minutes at 37 °C, digestion was stopped by incubation with 1mM Pefabloc SC for 5 minutes and samples were subsequently incubated with 4 M Guanidine thiocyanate (Gnd, Sigma-Aldrich, UK) for 10 minutes

at RT with shaking. The cells were then washed and blocked with blocking solution 3% BSA in D'PBS, and incubated with primary antibodies to PrP in blocking solution for 30 minutes at 37 ^{o}C and then 30 minutes at RT with shaking at 60 rpm in an orbital shaker. Alexa 488-conjugated secondary antibody was used for 60 minutes followed by counterstaining with DAPI for 15 minutes. The slides were mounted with Vectashield mounting media and examined by confocal microscopy.

2.12.4 Immunocytochemistry of sub-cellular localisation by double labelling

Cells intended for double immunostaining (PrP and one of following proteins: GFAP, NANOG, EEA1, giantin, calnexin, LAMP1, LAMP2b, rab11A, caveolin1, and clathrin) were washed, fixed and permeabilised, (denaturated if desired, as described in the section 2.12.3). Immunostaining for PrP was performed as described previously in section 2.12.2). The cells were then subsequently incubated with an antibody against the second cell protein overnight at 4 ^{o}C with shaking at 60 rpm in an orbital shaker, followed by the Alexa 546-conjugated secondary antibody incubation for 60 minutes. The cells were counterstained with DAPI for 15 minutes. The slides were mounted and examined by confocal microscopy.

2.12.5 Immunocytochemistry "pulse/chase" experiments

The processing of cells for immunostaining in the PrP pulse/chase experiments was carried as described previously (sections 2.12.2 or 2.12.3) except that no primary PrP antibody incubation step was performed and the cells were processed directly to blocking and labelling with Alexa 488. Then samples were incubated overnight at 4 ^{o}C, with shaking with one of following primary antibodies: EEA1, giantin,

calnexin, LAMP1, LAMP2b, rab11A, caveolin1, and clathrin, followed by incubation with the Alexa 546-conjugated secondary antibody for 60 minutes. The nuclei were courterstained with DAPI for 15 minutes. The slides were mounted with Vectashield mounting media and examined by confocal microscopy.

2.13 CONFOCAL MICROSCOPY

Immunostained cultures were examined at 23°C using a laser scanning confocal microscope Nikon Eclipse TE2000-U with a 40X 0.75 Plan Fluor DIC M/N2 dry and 60X 1.40 Plan Apo VC oil objectives. Images were exported via EZ-C1 Gold version 3.30 software in an 8-bit tagged image file format. The entire hESC study was analysed with an LSM5 Pascal laser scanning confocal Zeiss microscope.

Image files were processed with ImageJ (National Institute of Health) and Photoshop (Adobe) programs without altering settings for brightness/contrast or any other colour manipulations.

2.14 QUANTITATIVE IMAGE ANALYSIS AND STATISTICAL ASSESSMENT OF THE PrP^{Sc} COLOCALISATION DATA

Two methods were used to test the true colocalisation of PrP^{Sc} with cell organelles. The first was a pixel-based method in which the observed amount of green and red pixel colocalisation was compared to the value expected to arise if the colour pixels were uniformly randomly distributed. The statistical significance of the result was then determined.

In the second method colocalisation was assessed using the particle-based approach of Bolte and colleagues (Bolte *et al.*, 2006), which assesses the distance between the

centres of mass of particles in the image. Manders' M_1 coefficient (Manders, *et al.*, 1992) was then calculated. Both methods were applied to representative examples of the data generated in the immunocytochemistry experiments.

2.14.1 Pixel-based colocalisation analysis

The analysis was carried out on three individual images of each time point from one experiment. The pixel colocalisation analysis was carried out on three pictures (containing 1 to 4 cells) in which HK cells had been exposed to vCJD brain homogenate and then double immunostained for markers of early endosomes, Golgi complex or lysosomes and PrP^{Sc} at 1h, 24h, 48h, 72h post-exposure (36 images in total). The endoplasmic reticulum data was not included in the analysis because the immunostaining of this cellular organelle was too weak to bear quantitative colocalisation analysis.

Analysis of colocalisation of the exogenous PrP^{Sc} within the cellular organelles was carried out using the ImageJ program, "Colocalisation Analysis" plugin (National Institute of Health, USA). The method evaluates observed colour pixels for cellular organelles (red) and PrP^{Sc} (green) and cell nuclei (blue) from the total pixel value. The measured pixel values enabled the calculation of the expected random distribution of colocalisation measure and the observed colocalisation of green (PrP^{Sc}) and red (cell organelle) pixels.

The observed colocalisation was measured as follows:

1. Open picture in ImageJ→Image→Colour→Split channels

- Red channel picture→Analyse→Histogram→Sum all red pixel values

- Green channel→Analyse→Histogram→Sum all green pixel values

Values for Mean and Standard deviation (Σ) were recorded to calculate Fay's threshold = Mean + 2xΣ, following (Fay *et al.,* 1997). This is a heuristic for setting a threshold for each channel that aims to eliminate background noise. It was used to calculate observed colocalisation values for all images.

2. Plugin→Colocalisation analysis→Colocalisation finder→OK

A picture will reflect colocalisation points in a correlation diagram. The colour signal thresholds were then adjusted according to value calculated by the Fay's formula. The plugin then evaluated observed colocalisation of the red and green pixels.

The expected colocalisation was calculated as follows:

1. The value of total red and total green pixels was measured in each channel. The % of red pixels was calculated as the total red pixels/total number of pixels. The % of green pixels was calculated as the total green pixels/total number of pixels.

2. The value of expected random distribution of colocalisation was calculated as the % of red pixels multiplied by % of green pixels.

The statistical significance of the results was assessed by making the *null hypothesis* that the red and green pixels are randomly uniformly distributed. This implies that the number of colocalised pixels follows a binomial distribution. This allows calculation of the P-value, the probability for the observed or greater value arising from the null hypothesis. The P-values for each image were calculated using Excel (Binom.dist function) and checked using the open-source statistical package R (using the binom.test function).

2.14.2 Particle-based colocalisation analysis

The analysis was performed on the same data set as the previous pixel-based analysis (early endosomes and lysosomes). The particle-based technique is particularly well suited to these structures because identifying them can be done automatically by the software and does not require complex image segmentation or edge detection. However, the same method cannot be applied to Golgi complex data because the software was not able to identify the centres of mass for more complex morphological structures such as the Golgi complex.

The colocalisation of PrP^{Sc} (green) within cell organelles (red) was measured using the ImageJ plugin "Just another Colocalisation Plugin" (JACoP) as follows:

1. Open picture in ImageJ→Image→Colour→Split channels

2. Plugins→Jars→JACoP

- Particle size and signal threshold was measured by framing of investigated particles (Figure 2.7). Analyse→Measure. The Mean for each channel revealed the signal threshold the investigated particles are corresponding to and the size of the investigated particles (in pixels).

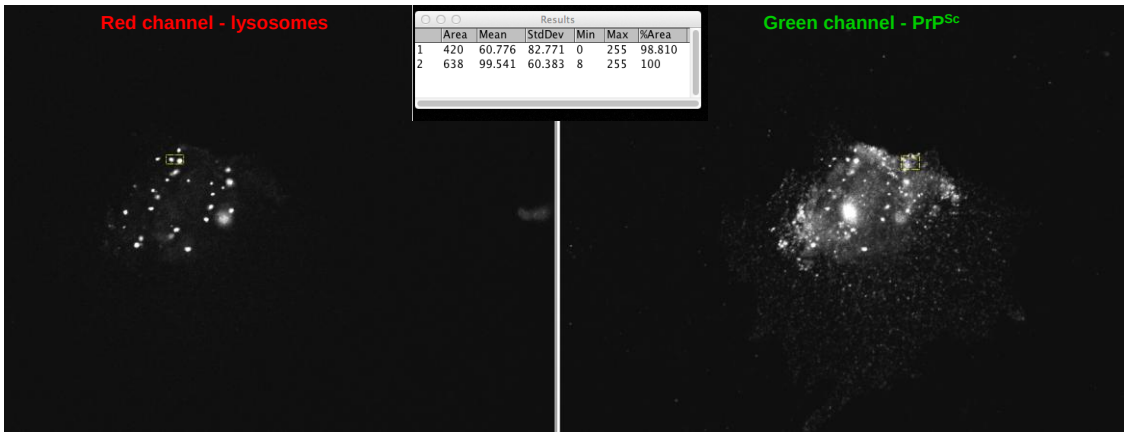


Figure 2.7: Example of framing the investigated particle to measure its size and signal threshold

Then the signal threshold and the size (usually 10-100 pixels) of the investigated particles were set according to these measured values (Figure 2.8).

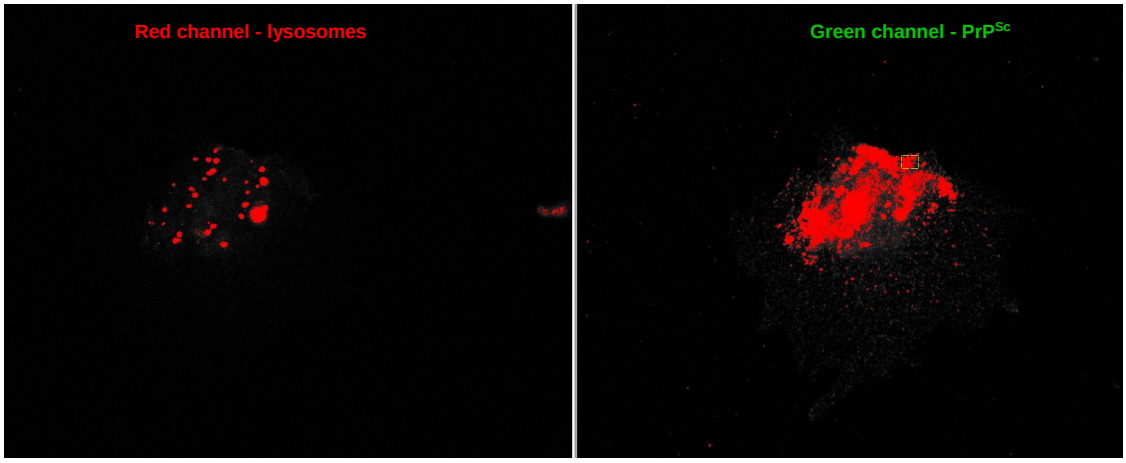


Figure 2.8: The signal threshold and the size of the investigated particles were set according to measured values

The JACoP features allow a set of commonly used co-localisation indicators such as Pearson's coefficient, Manders' coefficient, the overlap coefficient, and so on to be calculated. The plugin also produces a centres map of the colocalised particles and a log containing the calculated values.

A rigorous statistical analysis of the particle colocalisation would require developing a detailed mathematical understanding of the two-dimensional spatial statistics of both lysosome and endosome structures, which is beyond the scope of the present study. A literature search failed to identify such a tool.

3. RESULTS

3.1 CHARACTERISTICS OF THE CELL CULTURES USED IN THE STUDY

3.1.1 Objectives

To confirm the *PRNP* codon 129 genotype, characterise the morphology and evaluate PrP^C expression in the cells used in this study.

3.1.2 Rationale

Thus far, the mechanisms involved in cellular susceptibility and human prion replication have not been studied in cultured human cells due to the apparent absence of any well characterised human cells susceptible to human prion infection *in vitro*.

The histopathological, biochemical and epidemiological evidence suggest that the variant Creutzfeldt–Jakob disease (vCJD) is most likely to be a result of oral ingestion of the bovine spongiform encephalopathy (BSE) agent (Will *et al.*, 1996; Collinge and Rossor, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997a; Prusiner, 1998). Cells of the lymphoreticular system, specifically FDCs and B lymphocytes, are also known to be involved in prion pathogenesis (Fraser and Farquar, 1987; Fraser *et al.*, 1989; McBride *et al.*, 1992; Klein *et al.*, 1997; Brown *et al.*, 1999; Montrasio *et al.*, 2000; Weissmann *et al.*, 2001) and there is also a great deal of evidence to suggest that this is also true in vCJD (Kitamoto *et al.*, 1991; Hilton *et al.*, 1998; reviewed in Mabbott and MacPherson, 2006). This suggests that the FDC-like HK cells, derived from human tonsils and having FDC-like characteristics, are a potentially useful cell line to study cell response to exposure of infectious prions.

In human prion diseases the phenotype, susceptibility and incubation period are all governed, in part, by host genetics, in particular by the methionine/valine (MV) polymorphism at codon 129 of the human prion protein gene (*PRNP*) (Collinge and Alpers, 2006; Bishop *et al.*, 2006; Parchi *et al.*, 2009; Mastrianni, 2010). PrP^C expression levels also affect susceptibility (Bueler *et al.*, 1993).

As the aim of this study was to directly examine the cellular response of extraneuronal lymphoreticular FDC-like HK cells and undifferentiated human embryonic stem cells to exposure to infectious prions from human (variant, sporadic, iatrogenic Creutzfeldt-Jakob disease) and animal (bovine spongiform encephalopathy) brains, analysis of the cell lines for their *PRNP* codon 129 polymorphism and level of PrP^C expression was an important consideration. Thus *PRNP* codon 129 genotype is an indicator of relative cell susceptibility and PrP^C an obligatory prerequisite for cells to replicate infectious prions. Hence determining the *PRNP* polymorphic status and the level of PrP^C expression of the studied cells were matters of great interest.

Determining the *PRNP* codon 129 genotype of seven hESC lines available in this study (Table 3.1) was necessary in order to select representative lines of each genotype for *in vitro* challenge experiments. The results were as follows:

PRNP codon 129 genotype	Norman population frequency[1]	hESC line results	Identity of the hESC lines tested[2]
MM	44%	3/7 (43%)	RCM-1, RH3, RH4
MV	44.5%	3/7 (43%)	RH1, RH5, RH7[3]
VV	11.5%	1/7 (14%)	RH6

Table 3.1 *PRNP* codon 129 polymorphisms of seven hESC lines available for the study

[1] - Data published in Bishop *et al.*, 2009

[2] - Isolation described in Fletcher *et al.*, 2006 and De Sousa *et al.*, 2009

[3] - Showing a non-pathogenic 24 bp deletion of the 129-M allele

The representation of the three common polymorphic groups among the seven tested hESC was found to be consistent with that of the general UK population as a whole (44% MM, 44.5% MV, 11.5% VV) (Bishop *et al.*, 2009). However one cell line was identified to carry deletion on one of the *PRNP* 129-M alleles, and accordingly was not investigated further.

3.1.3 Experimental results

Three hESC cell lines, differing in their *PRNP* codon 129 genotype, were selected for the study: RCM-1 (homozygous for methionine), RH1 (heterozygous), and RH6 (homozygous for valine). Unfortunately, the only VV hESC line available to this study proved to have very slow growth and showed poor viability in culture. Although it would be interesting to obtain data on all three hESC lines differing in their *PRNP* codon 129 genotype, the RH6 line would have presented considerable practical difficulties and possibly yielded incomparable results with the two other hESC cell lines. Consequently, the study focused on RCM-1 (MM) and on RH1 (MV) hESC lines, both originating from clinically failed eggs rescued by parthenogenetic activation.

Interestingly, despite the fact these two normal euploid and biparental pluripotent hESC lines were not genetically related, they revealed the closest match in their expression of markers of an undifferentiated state. This phenomenon is most likely to result from their method of isolation. Moreover, because of the known susceptibility of methionine homozygous individuals to the BSE/vCJD agent and because the *PRNP* codon 129 heterozygosity is the most frequently occurring genotype in the general UK population, the choice of one MM and one MV representative cell line

seemed sufficient for the purposes of this study. The available follicular dendritic cell-like cell line (HK) was found to be VV somewhat completed the repertoire to be able to investigate the cell susceptibility to prion in regards to *PRNP* codon 129 genotype.

3.1.3.1 PRNP codon 129 genotyping of the cells used throughout the study

To confirm the polymorphism at the codon 129 of the prion protein gene (*PRNP*), the high molecular weight DNA was extracted from the cell pellets of the hESC RCM-1 and RH1 lines and the FDC-like HK cell line as described in section 2.5.1 and a fragment containing the *PRNP* coding sequence was amplified by PCR as described in section 2.5.2. The PCR products were visualised by agarose gel electrophoresis and SYBR green gel staining (Figure 3.1). Successful amplification was observed in all three studied cell lines: the hESC of the RCM-1 (lane 1) and RH1 (lane 2) cell lines and the FDC-like HK cells (lane 3). The PCR products for the three different codon 129 polymorphisms (lane 4-6) served as a positive control for the PCR reaction process.

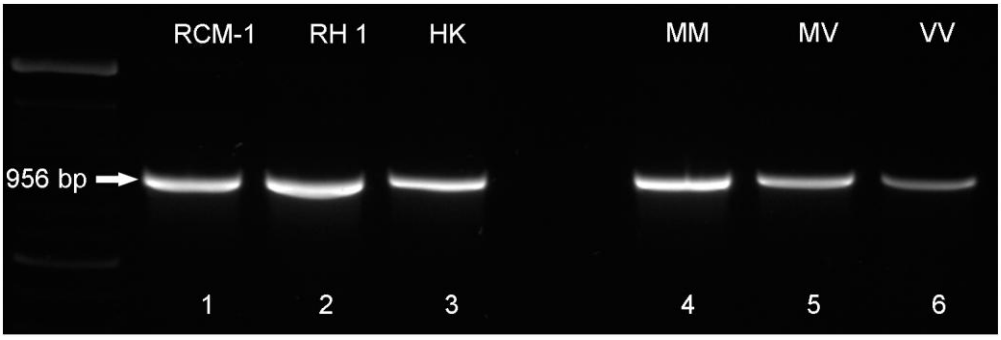


Figure 3.1: PCR amplification of the *PRNP* gene sequence
DNA from the hESC RCM-1 (lane 1), RH1 (lane 2) cell lines and the FDC-like HK cells (lane 3) was obtained by cell pellet lysis and column purification using the DNA Blood Mini Kit according to manufacturer instructions. The amplification of the *PRNP* gene sequence by PCR was performed as described in section 2.5.2. PCR of the control *PRNP* gene sequence samples (lane 4-6) served as a positive control for the PCR reaction. Detection of the PCR products was performed by agarose gel electrophoresis and SYBR Green gel staining using the ChemiDoc XRS imaging system. The DNA mass, in bp, is marked left.

The amplified DNA was then digested with restriction enzyme NspI (Figure 3.2) cleaving the amplicon at *PRNP* codon 155 and at codon 129 only when the latter sequence coded for valine (-GTG-). The restriction fragment length polymorphisms (RFLP) allowed discrimination of the three genotypes of the studied cell lines RCM-1 (lane 1), RH1 (lane 2) and HK (lane 3) by agarose gel electrophoresis and SYBR Green gel staining. The positive controls for the *PRNP* codon 129 genotype MM (lane 4), MV (lane 5) and VV (lane 6) also served as a positive controls for the restriction digest. Comparison of the results obtained using the cell lines with the positive controls confirmed that the hESC of the RCM-1 line was of the *PRNP* codon 129 genotype MM (lane 1), the RH1 line was of the *PRNP* codon 129 genotype MV (lane 2) and the FDC-like HK cell line was VV (lane 3).

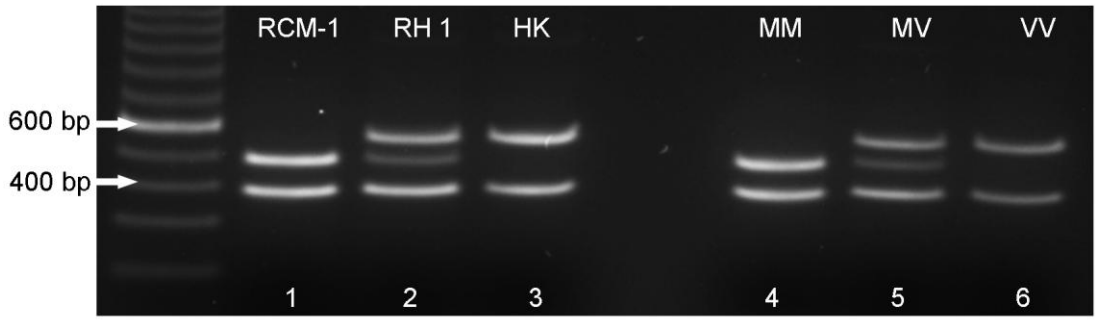


Figure 3.2: RFLP analysis of the *PRNP* codon 129 polymorphism
Confirmation of the *PRNP* codon 129 genotypes of the hESC of RCM-1 (lane 1) and RH1 (lane 2) cell lines and the FDC-like HK cells (lane 3) was performed by restriction enzyme digestion at 37 °C with NspI allowing discrimination of the three genotypes paradigms MM, MV and VV by agarose gel electrophoresis and SYBR Green gel staining. The controls for each *PRNP* codon 129 polymorphism type: MM (lane 4), MV (lane 5) and VV (lane 6) also served as a positive control of the digestion reaction. The DNA ladder, in 100 bp fragments, is marked left.

3.1.3.2 Morphological characterisation of the cells used in the study

Morphological analysis of the cultured cells is important in several respects. In a culture, cellular morphology can reflect the status of the cells, both in terms of the health of the culture and, in the case of cultured embryonic stem cells, it is an indicator of their state of differentiation. Undifferentiated stem cells are primitive cells with a regulated capacity for self-renewal, multilineage proliferation and differentiation, precise control of which *in vivo* is essential for development and tissue renewal. *In vitro* embryonic stem cells are heterogeneous, containing a self-renewing population (or populations) of cells but also showing evidence of low level, presumably random, differentiation.

These hESC RCM-1 and RH1 cell lines were established from whole blastocysts without exposure to animal immune complement to recover embryo inner cell mass, on an extracellular matrix substrate of purified human laminin with transitional reliance on mitotically inactivated human fibroblast feeder cells. Isolation of these cells was the first example of a new derived hESC without direct exposure to any animal cell products.

Cultured cells were readily observed in their native state using differential interference contrast microscopy (Figure 3.3). Undifferentiated hESC of RCM-1 (A) and RH1 (B) cell lines shared a common morphology in forming densely packed round colonies with clear margins. Based on the location of the cells in colonies, different morphological features could be identified. Cells at the centre of the colony were rounded and lay tightly packed against each other, suggesting a relatively an undifferentiated state and close cell membrane contact. Compared to the cells at the core, stem cells at the periphery of these masses appeared to be in the initial stage of cytodifferentiation as judged by their flattened morphology (arrows). The cells were

maintained as described in section 2.2.2. Cells were always plated one day before exposure to brain spiked medium. The passage numbers of the hESC used in the experiments varied between 45 to 65 for RCM-1, and 60 to 85 for RH1.

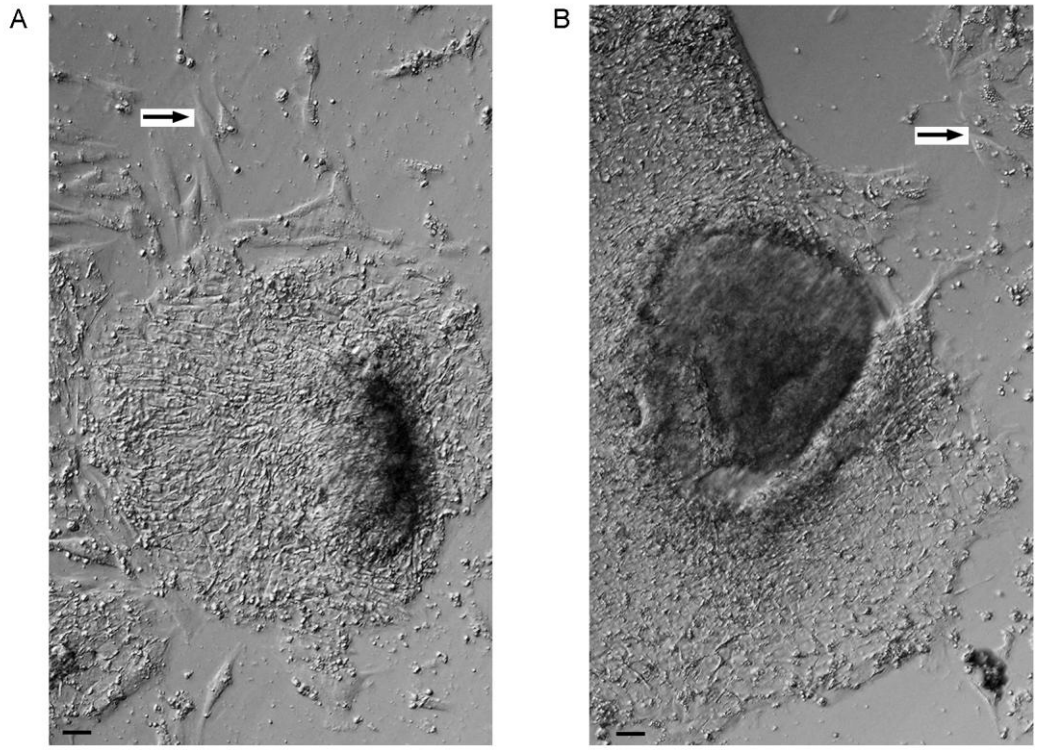


Figure 3.3: Morphology of human embryonic stem cells
Two day old culture of the hESC of RCM-1 (A) and RH1 (B) cell lines shared common morphology consisting of cells densely packed in round colonies surrounded by more flattened cells (arrows). The cultured hESC were observed by a differential interference contrast microscope. Scale bars represent 50 µm.

The extraneuronal lymphoreticular FDC-like HK cell line is a primary cell line derived from human tonsils (Figure 3.4). The cells were cultured in a complete cell culture medium supplemented with foetal calf serum (FCS) and antibiotics (as described in section 2.2.1). This serum was sourced from TSE-free herds in New Zealand that have been classified by the Medicines and Healthcare products Regulatory Agency (MRHA) and from comparable health agencies throughout the world. Therefore, it was unlikely to be contaminated by prions that could interfere

with results of this study. Passaging of the HK cells was performed roughly every 10 days and cells were always transferred to fresh flask or chamber slide one day before exposure to prions.

The cells in culture display a relatively homogenous morphology (A). They are large adherent cells displaying typically elongated and bipolar morphology with numerous cellular projections and slender cytoplasmic protrusions (B) and heterochromatic oval nuclei with conspicuous nucleoi. Once the HK cells underwent a certain number of passages (~10) they became senescent, stopped dividing, but remained viable.

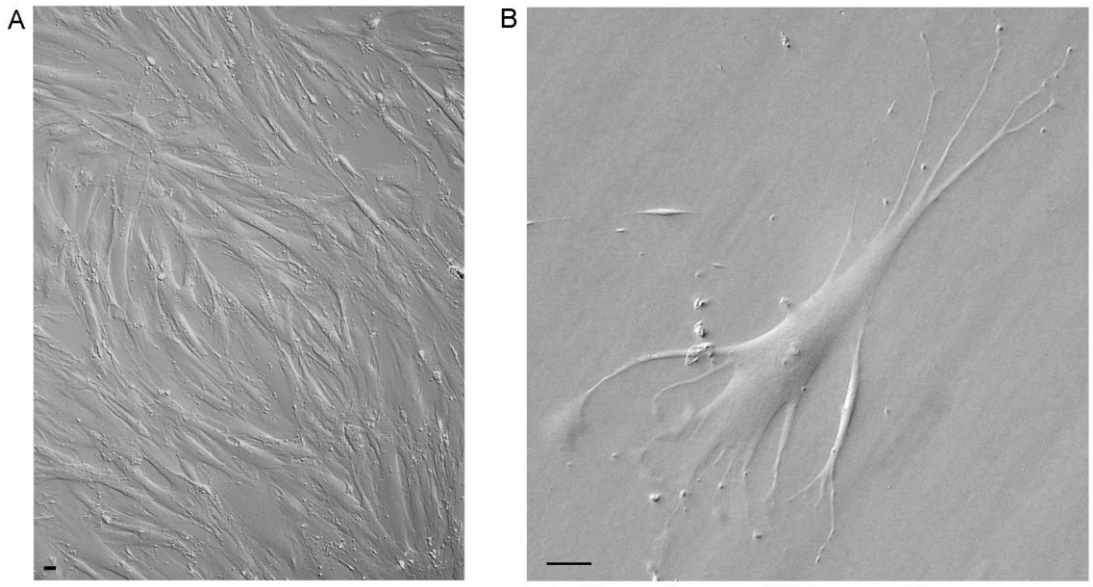


Figure 3.4: Morphology of the FDC-like HK cells
The extraneuronal lymporeticular HK cells derived from human tonsils by Dr Y.S. Choi (New Orleans) displayed morphology typically elongated and bipolar with numerous cellular projections. The cultured cells were observed by a differential interference contrast microscope. (A) Photograph of FDC-like HK cells in culture. (B) Single HK cell. Scale bars, 50 µm.

3.1.3.3 Expression of normal prion protein (PrP^C) in undifferentiated hESC of RCM-1 and RH1 lines and FDC-like HK cells

The analysis of the PrP^C expression was carried out by Western blot using mAb 3F4, an anti-PrP primary antibody recognising human PrP amino acid residues 109-112 (Figure 3.5). Pellets of the hESC RCM-1 (lane 1) and RH1 (lane 2) lines and the FDC-like HK cells (lane 3) were homogenised in extraction buffer to 10% w/v suspensions and the same volume of the cell lysates were loaded on the gel. The Western blot procedure was routinely performed as described in section 2.7.

The PrP^C expressed by undifferentiated hESC of RCM-1 (lane 1) and RH1 (lane 2) was not detectable by Western blot under the conditions used. In contrast, HK cells (lane 3) appeared to express abundant amounts of prion protein with the expected molecular weight of around 30 kDa.

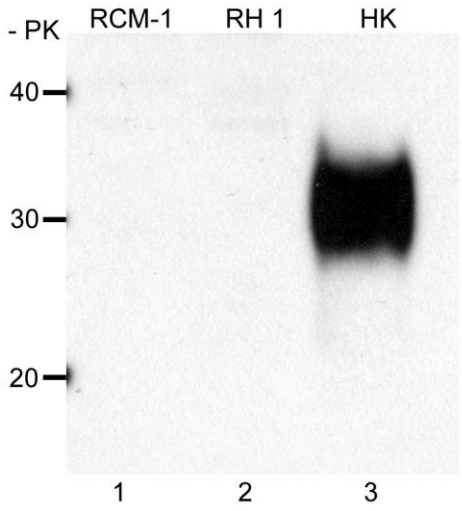


Figure 3.5: Western blot analysis of PrP^C expression level in hESC and FDC-like cells
The hESC of RCM-1 (lane 1) and RH1 (lane 2) cell lines and the FDC-like HK cells (lane 3) were cultured until confluent before harvesting for Western blot analysis. The cell pellets were homogenised in extraction buffer to 10% w/v suspensions and same amounts of the cell lysates mixed with loading buffer were loaded onto gel. The immunobloting procedure was carried out according to the standard method as described in section 2.7 and the PrP^C was detected using 3F4 monoclonal antibody. The molecular weight, in kilodaltons (kDa), is marked left.

The level of PrP^C expressed by HK cells was compared to the level of PrP^C present in a normal brain from the World Health Organisation (WHO) (Minor *et al.*, 2004) (Figure 3.6). Densitometric evaluation of the PrP^C signal (method described in section 2.10.5) revealed that the HK cells (lane 5) express 1.3 times more of PrP^C than is present in the normal brain (WHO, lane 1) as equal volumes of the 10% w/v suspensions were loaded onto gel in these two lines. Doubling dilution series of the WHO standard brain are shown in lanes 2-4.

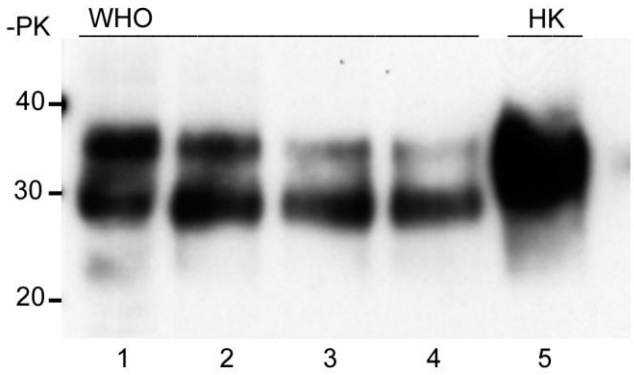


Figure 3.6: Comparison of PrP^C expression level in normal brain and HK cells
Level of PrP^C in the 10% WHO normal brain standard (lane 1-4, doubling dilution series lane 2-4) and 10% HK cell lysate (lane 5) were analysed by Western blot using 3F4 monoclonal antibody. WHO standard (lane 1) and HK cell lysate (lane 5) contain equal volumes of loaded proteins (w/v). The molecular weight, in kDa, is marked left.

3.1.4 Summary

- The available human FDC-like cell line (HK) was found to be VV.
- The human embryonic stem cell lines chosen for this study were of the MM (RCM-1) and MV (RH1) genotypes at codon 129 of *PRNP*. The only line with a VV genotype found in the seven available hESC lines had poor growth characteristics and therefore was not included in these studies.
- The HK cells isolated from human tonsils are large adherent cells displaying typically elongated and bipolar morphology with numerous slender cytoplasmic protrusions.
- hESC formed rounded colonies of tightly packed cells suggesting an undifferentiated state, surrounded by spontaneously differentiating flattened cells at the periphery.
- The level of PrP^C expression in the RCM-1 and RH1 lines seems to be below detection by Western blot when compared to the abundant PrP^C expression level of the FDC-like HK cells.

3.2 PROTEIN MISFOLDING CYCLIC AMPLIFICATION

3.2.1 Objectives

To investigate potential for conversion of PrP^{C} from HK cells into its pathologic PrP^{Sc} isoform and to determine whether these cells have the minimal required components to support PrP^{Sc} formation.

3.2.2 Rationale

Prior to performing the actual attempts for cell culture infection with prions, it was of interest to test whether HK cells have all the required components to propagate PrP^{Sc}. The method chosen to determine this is called protein misfolding cyclic amplification (PMCA) (described in section 2.8). Previous successful PrP^{Sc} amplification by PMCA using substrate PrP^{C} contained in brain homogenates (Saborio *et al.*, 2001; Soto *et al.*, 2002; Castilla *et al.*, 2005a) suggests that PrP^{C} obtained from cell culture lysate may also support PrP^{Sc} formation (Castilla *et al.*, 2006).

3.2.3 Experimental results

3.2.3.1 Preparation of the substrate and seeds for PMCA

In order to determine whether HK cell PrP^{C} can be converted to PrP^{Sc} and therefore also whether HK cells have the minimal required components for PrP^{Sc} formation, a PMCA experiment was performed on HK cell homogenates seeded with iCJD, sCJD, BSE and vCJD brain homogenates. For optimal PMCA efficiency, aspects of both seed and substrate preparation had to be carefully considered (Castilla *et al.*, 2005a; Castilla *et al.*, 2005b; Castilla *et al.*, 2006). The most important aspects included the

composition of the conversion buffer (Castilla *et al.*, 2006), the choice and preparation of the substrate (material stored exclusively at -80 °C), which had to be prepared just before setting up the reaction (Figure 3.7; A), and titration of the PrP^{Sc} seeds to a level detectable at the lower end of the linear range of a standard Western blot after limited PK digestion (Jones *et al.*, 2009). The seed titration step (Figure 3.7; B) allowed us to asses the degree of newly amplified PrP^{Sc} by PMCA. Therefore, the HK cell culture substrate was always freshly prepared directly prior the PMCA reaction (described in section 2.8.1) and the brain homogenate used for seeding the reaction was added to the reaction at amount just detectable by Western blot (described in section 2.8.2) as shown (Figure 3.7; B, lane 5, 8, 11, 14) so that all PMCA reaction contained approximately equivalent amount of PrP^{Sc}.

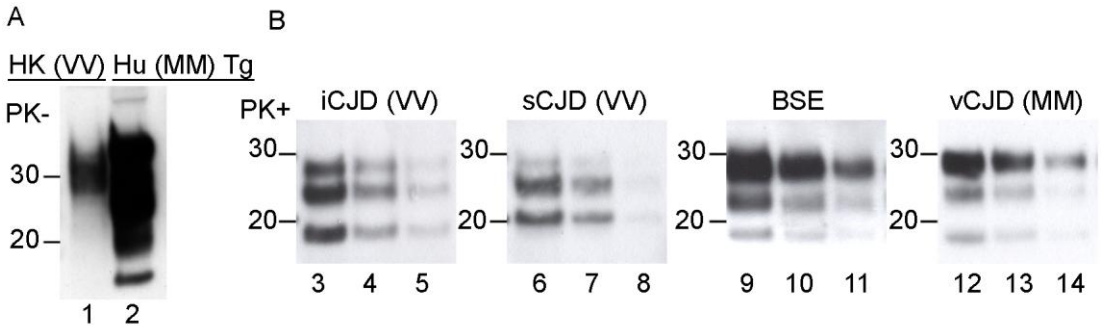


Figure 3.7: Western blot analysis of substrate and dilution series of seeds for PMCA
(A) Western blot analysis of the substrates for PMCA: the PrP^{C} level contained in 7% w/v HK cells lysates (lane 1), the PrP^{C} level contained in 10% w/v Hu (MM) Tg mouse brain homogenate (lane 2). In both cases 1.5 µl of substrate suspension was loaded onto the gel. Western blot was performed by the standard method and the PrP^{C} was detected with the monoclonal antibody 6H4. (B) Doubling dilution series of the brain homogenates iCJD (lane 3-5), sCJD (lane 6-8), BSE (lane 9-11), vCJD (lane 12-14) – seeds used in the PMCA reaction. Samples were PK treated at a PK concentration of 50 µg/ml and the presence of PrP^{Sc} was detected using 6H4 monoclonal antibody. The molecular weight, in kDa, is marked left on each blot.

3.2.3.2. PMCA experiment

It is important to mention that the HK cell lysate used as a substrate for PMCA (Figure 3.7; A, lane 1) reaction was not supplemented with a PrP inert substrate such as $PrP^{0/0}$ brain homogenate in putative order to enhance the level of amplification (Castilla *et al.*, 2006), as our goal was to determine whether the HK cells themselves have all the required components to sustain PrP^{Sc} replication. The reaction mixtures were prepared at a seed-to-substrate ratio of 1:7 and the mixtures were divided into two duplicates. The control (unamplified aliquot) was immediately frozen at -80 °C and the PMCA aliquot was subjected to the PMCA reaction. The experiment reaction consisted from 48 cycles of 40 seconds sonication at 80% total power output and 29 minutes 20 seconds incubation at 37 °C for 24 hours.

Positive PrP^{Sc} amplification was observed when iCJD and sCJD seed was used (codon 129 VV) (Figure 3.8; lane 2 and 4), whereas no PrP^{Sc} amplification could be detected in the case of BSE and vCJD (codon 129 MM) (Figure 3.8; lane 6 and 8). The successful amplification may be due to the prion protein compatible 129 codon genotype of these two cases with the 129 codon genotype of the HK cells (VV type) (Jones *et al.*, 2007). The AD brain (MM type) homogenate was used as a negative control (Figure 3.8; lane 9-10).

This PMCA result is a representation of three independent and identical experiments.

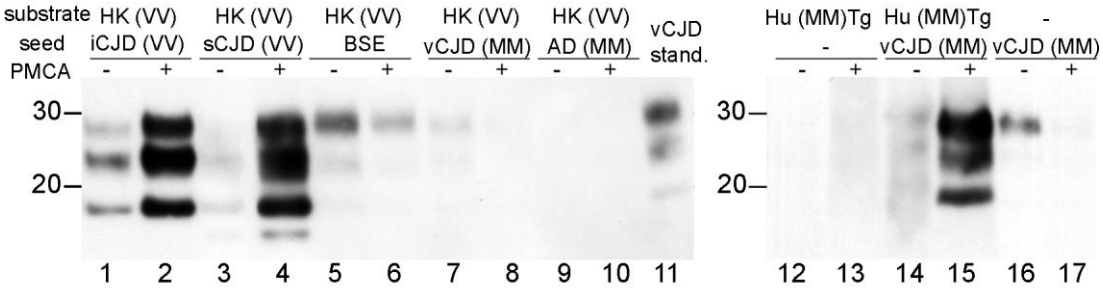


Figure 3.8: Western blot analysis of PMCA using HK cell culture as a substrate
Each type of seed has an un-amplified control sample (lane 1, 3, 5, 7, 9, 12, 14, 16) and a sample subjected to 48 cycles of PMCA (lane 2, 4, 6, 8, 10, 13, 15, 17) (described in section 2.8.3). AD was used as a negative control (lane 9-10). All samples were subjected to PK digestion (50 µg/ml for 60 minutes at 37 °C) prior Western blot analysis performed by the standard method and the PrP^{Sc} was detected with the 6H4 monoclonal antibody (lanes 1-11) and 3F4 monoclonal antibody (lanes 12-17). The vCJD 2B standard (lane 11) was used as a control of PrP migration and blotting procedure. The marked molecular weight is in kDa.

The vCJD brain seed (*PRNP* codon 129 genotype is MM) in transgenic humanised MM mouse brain substrate (Tg Hu MM) (*PRNP* codon 129 genotype is also MM) (Figure 3.8; lane 15) was used as a positive control for the PMCA reaction, alongside an appropriate negative control samples (Figure 3.8; lane 13 and 17). Negative controls, as expected, lacked amplification (Colby *et al.*, 2007) in the unseeded Tg Hu MM mouse substrate (Figure 3.8; lane 13) and also no amplification could be observed in the seeded sample lacking brain homogenate substrate (Figure 3.8; lane 17).

3.2.4 Summary

- The extracts from the extraneuronal, lymphoreticular, FDC-like HK cells support PrP^{Sc} amplification in PMCA reaction, in a *PRNP* codon 129-depended manner.
- Whether PMCA could be viewed as a rapid indicator of a cell type's potential susceptibility to prion infection *in vitro* has been tested in following assay by directly challenging HK cells *in vitro* with some of the brain homogenates used to seed the above PMCA reactions (section 3.3).

3.3 INFECTION STUDIES OF HK CELLS

3.3.1 Objectives

To evaluate whether the extraneuronal lymphoreticular FDC-like HK cells are capable of PrP^{Sc} replication *in vitro* and PrP^{Sc} propagation in the longer term.

3.3.2 Rationale

The known involvement of FDCs in variant CJD and the high levels of PrP^{C} expressed by HK cells indicate that these cells may be susceptible to prion infection and competent to propagate that infection *in vitro*. HK cells were therefore exposed to infectious brain homogenates taken from a variety of prion infected brain sources, prepared by different methods and exposed under different culture conditions. Human CJD brain homogenates were used to exclude the possibility that the so called "species barrier" would prevent infection. The experimental set up also provided the opportunity to infect HK cells using brain homogenates from cattle BSE, which is the most likely source of variant CJD infection in humans and for which species specific PrP antibodies exist.

3.3.3 Experimental results

3.3.3.1 PrP^{res} analysis of brain tissue used for infection studies

Western blot analysis served as confirmation of the distinct prion agents as well as an evaluation of the level of PrP^{res} in the brain homogenates used for cell exposure prior each *in vitro* infection study. The presence of PrP^{res} was confirmed by partial proteolytic degradation of all brain homogenates and the characteristic PrP^{res}

glycosylation patterns associated with different prion diseases and phenotypes were confirmed by Western blotting. The 10% w/v brain homogenates were digested with PK for 60 minutes at 37 °C. Western blots (Figure 3.9) showing levels of total PrP (samples without PK treatment) (lane 1-4) and levels of PrP^{res} (samples after treated with PK) (lane 5-8). Two anti-prion monoclonal antibodies 3F4 (Figure 3.9; A) or 6H4 (Figure 3.9; B) recognising different epitopes were used. The PrP^{res} type nomenclature of Parchi and Gambetti (Parchi *et al.*, 1997) was used throughout.

The AD brain homogenate (Figure 3.9; lane 1 and 5) was used in the study as a negative control for presence of PrP^{res} and as a positive control of any changes in cell morphology caused by exposure to brain tissue components. The human growth hormone associated iCJD (Figure 3.9; lane 2 and 6) used in the cell infection studies was of the same *PRNP* codon 129 genotype as the HK cell culture (VV). Western blot analysis of PrP^{res} (lane 6) showed this case to be type 2A. The anti-PrP antibody 3F4 identifies human PrP, but not bovine PrP, whereas the anti-PrP antibody 6H4 recognizes both human and bovine PrP. The 3F4 antibody binds strongly to human and hamster PrP with specific requirement of two methionine residues at positions 109 and 112 in the human PrP (Lund *et al.*, 2007). Bovine PrP constitutes of different epitope at these corresponding positions. Brain stem homogenate from a case of bovine spongiform encephalopathy (BSE) was not recognised by 3F4 (Figure 3.9; A, lane 3 and 7), whereas the 6H4 antibody (Figure 3.9; B) recognised PrP (lane 3) and showed the characteristic predominance of di-glycosylated PrP^{res} (lane 7) in the BSE case. The predominance of the di-glycosylated band of PrP^{res} is a feature shared by BSE and vCJD. The vCJD brain homogenate was recognised by both 3F4 and 6H4 primary antibodies (Figure 3.9; A and B, lane 4 and 8). The migration

position of the non-glycosylated band of the PrP^{res} classified this case was type 2, and therefore the vCJD case was found to be, as expected, a type 2B (Figure 3.9; lane 8). These analyses confirmed that PrP^{res} was abundantly present in these brain samples and was of the expected PrP^{res} types. The findings also confirmed that the 3F4 antibody can be used to distinguish any newly formed PrP^{Sc} if human cells were to be successfully infected with BSE.

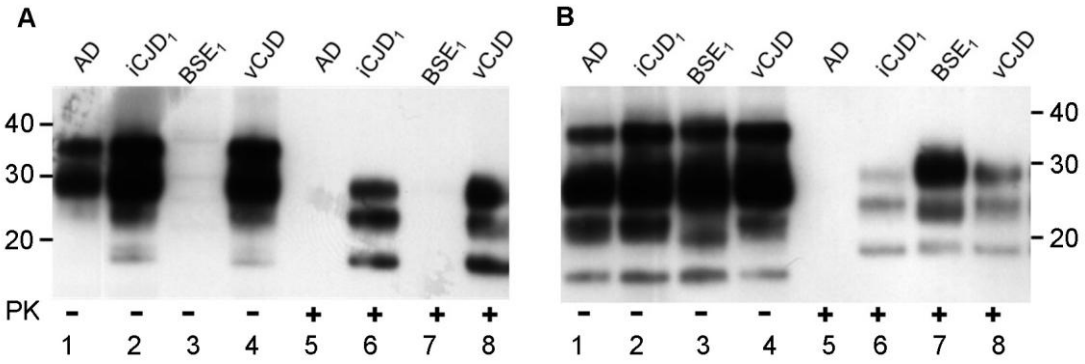


Figure 3.9: Western blot analysis of brain homogenates using two different anti-prion protein primary antibodies 3F4 and 6H4
Dissected brain tissues were homogenised in ice cold extraction buffer to 10% w/v brain suspensions and cleared from nucleocytoskeletal fraction by brief centrifugation. Samples were either allowed to remain undigested (lane 1-4) or digested (lane 5-8) with PK and loaded onto gel, as marked on the figure, at equal volumes. (A) Western blot was analysed with anti-prion monoclonal antibody 3F4 (recognising human, but not bovine, PrP amino acid residues 109-112). (B) Western blot was analysed with anti-prion monoclonal antibody 6H4 (recognising PrP residues 142-155). The molecular weight, in kDa, is marked on the sides.

The HK cells were plated 1-3 days prior exposure and after reaching 50-60% confluence, the cultures were exposed to brain material. This was to ensure that maximum cell surface would be exposed to brain homogenate, which might not have been achieved at a higher confluence, when cells are tightly packed together and have a more condensed morphology.

3.3.3.2 First set of HK cell challenges

In the first investigation to determine the HK cell's potential to propagate human prions, the cells were exposed to clarified suspensions of iCJD, vCJD and BSE brain homogenates. The AD case was used as a negative control. To avoid contamination by conventional, bacterial and fungal agents, a partial decontamination of the homogenates was attempted by heat pre-treatment or filtration as described in section 2.9.2.1. Each case was incubated with the cells at very low homogenate concentrations, with and without any pre-treatment (non-sterilised), to estimate the potential for possible toxicity and contamination depending on the origin of the homogenate, as well as the cellular response to exposure of crude brain material. The brain homogenates were diluted to 0.1% suspensions with complete (including serum) cell culture medium and incubated with cells for 24 hours. The brain spiked medium was then removed, cells were rinsed, given fresh (unspiked) culture medium and further maintained for 16 days, including one cell passage (split 1:2) and five medium changes. No obvious effects on cell morphology or growth rate were observed between challenged and unchallenged cell cultures. Interestingly, the non-sterilised brain homogenate did not result in noticeable bacterial, viral or fungal infection in these experiments.

The cells were then harvested for Western blot analysis using anti-PrP monoclonal antibody 6H4 (Figure 3.10). No detectable PrP^{res} accumulation was seen in lysates of cells challenged with iCJD, vCJD, or BSE. None of the brain homogenate pre-treatment seemed to have effects on cell susceptibility or to prion infection (Figure 3.10; lane 1-7).

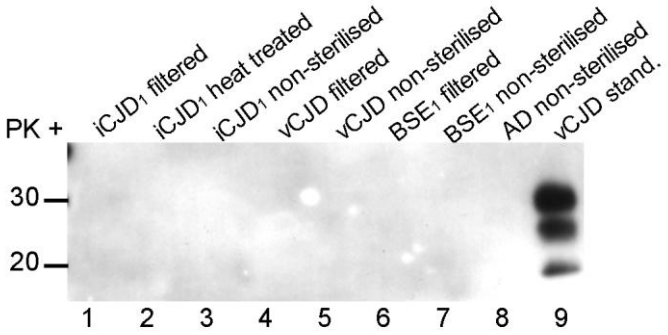


Figure 3.10: HK cells infection studies – first set
HK cells were exposed to 0.1% dilutions of $iCJD_1$, vCJD, BSE_1 or AD (control) brain homogenates which were either filtered (lane 1, 4, 6), heat pre-treated (lane 2), or used without pre-treatment (lane 3, 5, 7, 8). After an incubation period of 24 hours, the brain spiked medium was removed and cells were allowed to grow in fresh culture medium for 16 days with media changes every third day. The method is described in section 2.9.2.1. The cells were then harvested, PK treated and PrP^{res} collected by centrifugation as described in section 2.6.2.2 and subjected to Western blot analysis for PrP^{res} using primary antibody 6H4. The vCJD 2B standard (lane 9) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked on the left.

3.3.3.3 Second set of HK cells challenges

The methodology of the second attempt to infect the HK cells with prions (described in section 2.9.2.2) was modified by using three different concentrations of the $iCJD_1$ brain homogenate. This was an attempt to define the tolerance of challenged cells to brain homogenate diluted with medium as well as to investigate whether higher concentration of infectious brain homogenate will trigger prion infection of the HK cell culture. For this set of challenges, the growth hormone associated $iCJD_1$ brain (*PRNP* codon 129-VV), was selected to match the endogenous PrP^C genotype of the HK cells (which was also VV). In addition, the PrP^{res} of this $iCJD_1$ case was readily replicated *in vitro* by PMCA using the HK cell supernatant as a substrate for PrP^C to PrP^{res} conversion (Figure 3.8; lane 2). The objective was now to evaluate whether the challenged cells would replicate prion infection and therefore the positive *in vitro*

PrP^{res} amplification by PMCA could be used as an indication of cell culture susceptibility to infection with prions *in vitro*.

0.1%, 0.5%, and 1% dilutions of the brain spiked medium were treated with a very low concentration of Triton X-100 containing lysis buffer or extraction buffer to achieve partial extraction of the homogenates and to avoid excessive sticking of brain material on the cell surface. Some brain samples were also filtered or used without any pre-treatment to evaluate the contamination level (if any) caused by viruses or bacteria originating from the brain material. The AD brain homogenate was used as a negative control. Some HK cells were exposed to brain spiked medium when freshly passaged (Figure 3.11; marked *). This was to test whether changes in culture conditions would induce cells to become more vulnerable to infection with prions.

Cells were incubated with the brain spiked medium for 24 hours. The medium was then removed, the cells were rinsed, given fresh (brain-free) culture medium and further maintained for 26 days, which included one cell passage (split 1:2) and ten medium changes. The harvested cells were processed as described in section 2.6.2.2 and subjected to a routine Western blot analysis using anti-PrP monoclonal antibody 3F4 as described in section 2.7. Surprisingly, no cell-associated PrP^{res} could be observed at detectable levels in the HK cells challenged with the $iCJD_1$ homogenate in any of the treated conditions in this set of challenge (Figure 3.11). No obvious changes were observed in cell morphology caused by the use of higher brain homogenate concentrations or the buffer treatments employment, although the cell growth appeared to be slightly slower when compared to the previous experiment (First set; section 3.3.2). Contamination with conventional agents did not seem to be

an issue, even when using non-sterilised brain homogenates at 1% dilution. Therefore, the heat pre-treatment and filtration steps were omitted from subsequent experiments.

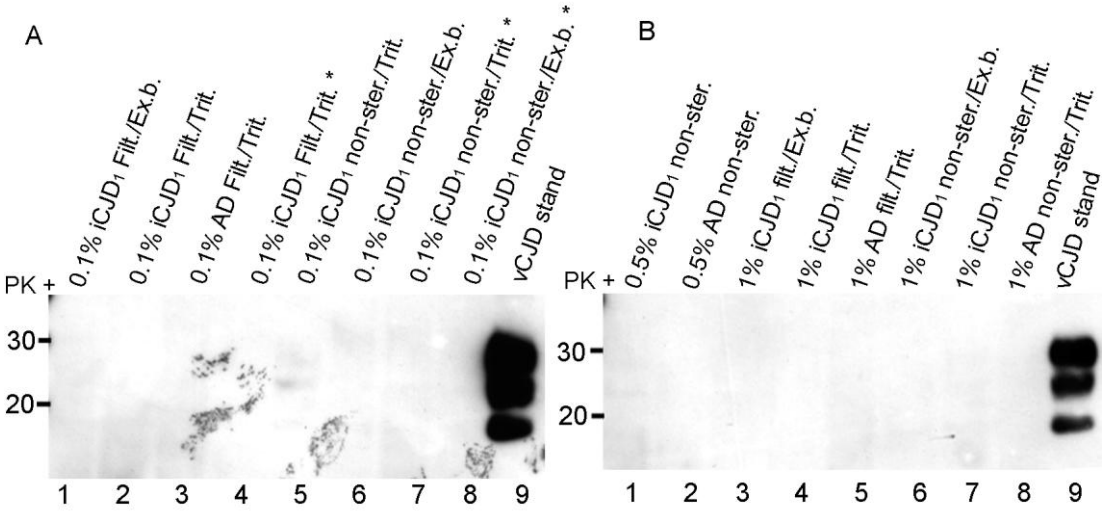


Figure 3.11: HK cells infection studies – second set
The HK cells were exposed to different dilutions of $iCJD_1$ and AD (control) brain homogenates: 0.1% (A), 0.5% (B, lane 1 and 2), or 1% (B, lane 3-8). These were either filtered (A lane 1-4; B lane 3-5) or used untreated (A lane 5-8; B lane 1, 2, 6-8) and additionally either treated with extraction buffer (A lane 1, 6, 8; B lane 3, 6) or triton lyses buffer (A lane 2-5, 7; B lane 4, 5, 7, 8) (as described in section 2.9.2.2). Cells were incubated with brain spiked medium for 24 hours. (*) Pellets of HK cells were gently resuspended in brain spiked medium and cells were plated into cell culture flask. After 24 hours, the brain spiked medium was removed, the cells were washed and allowed to grow in a fresh complete medium for 26 days, with medium changed 10x and one cell passage. When cells reached confluence, they were harvested and PK treated (as described in section 2.6.2.2) for Western blot analysis using the anti-prion protein primary antibody 3F4 to detect PrP^{res}. The vCJD 2B standard (lane 9) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.3.3.4 Third set of HK cells challenges

The third set of challenges employed another modification in pre-treatment of the brain homogenates prior to exposure of the HK cell cultures (section 2.9.2.3). The 10% w/v brain homogenates in a sterile 5% glucose solution were additionally homogenised by ribolysation and sonication as an attempt to break up aggregated

PrP^{Sc} multimers found in brain tissue and perhaps therefore to facilitate easier infection. Cells were analysed after incubation with three different dilutions of $iCJD_1$ brain homogenate – 0.1%, 0.5% and 2%. The AD case served as a negative control. HK cells were incubated with the brain spiked medium for 24 hours. The incubation period was in some cases prolonged to 68 hours. The purpose was to test whether the longer exposure time will trigger prion infection in these cells. Freshly passaged cells were also challenged (Figure 3.12; marked *). After the incubation period, the brain spiked medium was removed, the cells were rinsed, given fresh (unspiked) complete medium and cultured for additional 22 days, consisting in total of one cell passage (split 1:2) and eight medium changes. The cell lysates were then analysed for any cell associated PrP^{res} by Western blot using the 3F4 antibody (Figure 3.12).

Upon incubation with different dilutions of ribolysed and sonicated $iCJD_1$ brain homogenate PrP^{res} signal was detected in cell lysates of HK cells incubated with ribolysed and sonicated 0.5% $iCJD_1$ for 68 hours (Figure 3.12; lane 4) and 2% $iCJD_1$ for 24 hours (Figure 3.12; lane 6). However, the detection of PrP^{res} in cell lysates appeared to depend on the brain homogenate concentration used in cell challenge. As expected, PrP^{res} was not detected in the cell pellets from HK cells incubated with ribolysed and sonicated 0.1% and 2% AD brain material for 68 and 24 hours, respectively (Figure 3.12; lane 3 and 7).

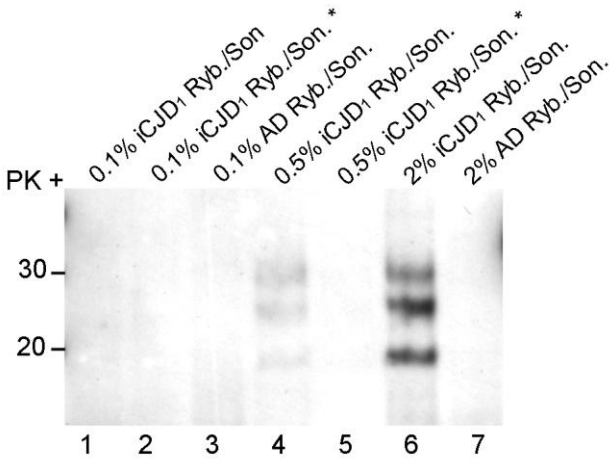


Figure 3.12: HK cells infection studies – third set
The HK cells were challenged with ribolysed and sonicated $iCJD_1$ and AD (control) brain homogenates diluted with complete cell culture medium to 0.1% (lane 1-3), 0.5% (lane 4, 5) and 2% (lane 6, 7). Cells were incubated with brain spiked medium for 68 hours (lane 1-4) or 24 hours (lane 5-7) as described in 2.9.2.3. (*)Pellet of HK cells was gently resuspended in brain spiked medium and cells were plated into cell culture flask. After exposure the brain spiked medium was withdrawn, cells were washed, given fresh complete medium and cultured for 22 days with medium changed eight times and one cell passage. Then the cells were harvested for PrP^{res} detection by Western blot analysis (as described in section 2.6.2.2) using primary antibody 3F4. The molecular weight (left) is in kDa.

3.3.3.5 Fourth set of HK cells challenges

Based on the PrP^{res} detection in cell lysate of HK cells exposed to ribolysed and sonicated 0.5% and 2% $iCJD_1$ and cultured for additional 22 days (Figure 3.12; lane 4 and 6), this set of cell challenges addressed the question whether the previously detected PrP^{res} (Figure 3.12) is newly produced, or represents remaining inoculum with a dose response effect. This set also investigated whether PrP^{res} from ribolysed and sonicated $iCJD_2$ (VV), $iCJD_3$ (MV), vCJD (MM), BSE_2 brain homogenates could cause prion infection of the challenged HK cells. The cells were exposed to a range of 0.1% - 2% brain homogenate dilutions (described in section 2.9.2.4, marked on the Figure 3.13) for 24 hours. Most of the cell infection studies are limited by the difficulty of specifically distinguishing the PrP^{res} of the inoculum from the PrP^{res}

newly produced in the infected cells. To assess whether PrP^{res} in HK cell lysates is newly formed or not, this set of challenges was designed as a time-course study (with the expanded times of culturing the cells, cell lysates were periodically analysed for presence of any PrP^{res} by immunoblot performed with the 3F4 monoclonal antibody). The advantage was taken of using BSE brain homogenate to infect cells and 3F4 primary antibody (recognising human but not bovine PrP) for detection of any newly formed PrP^{res} in HK cells. The experiment was carried out in duplicate. Cells from one flask were harvested for Western blot analysis, whilst cells in the other flask were split into two flasks and were allowed to grow until confluent, when the harvesting and splitting processes were repeated. This was repeated up to eight passages (Figure 3.13; A, B, C, D; only first four time points are shown). The immunoblot from the first time point (Figure 3.13; A) shows cell lysates analysed after 24 hours incubation of HK cells with brain spiked medium, followed by a PBS rinse and additional eight days cultivation, including one 1:2 cell passage and three changes of culture medium. Abnormal PrP in the PK digested cell lysates was detected in cells challenged with 2% $iCJD_2$ (VV) and 2% vCJD (MM) brain homogenates (Figure 3.13; A, lane 9 and 13, respectively). At the second (Figure 3.13; B) and third time points (Figure 3.13; C) the signal was reduced. PrP^{res} was further reduced after only a few passages and was not detected in cells exposed to BSE analysed by the 3F4 antibody, suggesting that the initial PrP^{res} signal in cell lysates was due to the residual material from the original input inoculum.

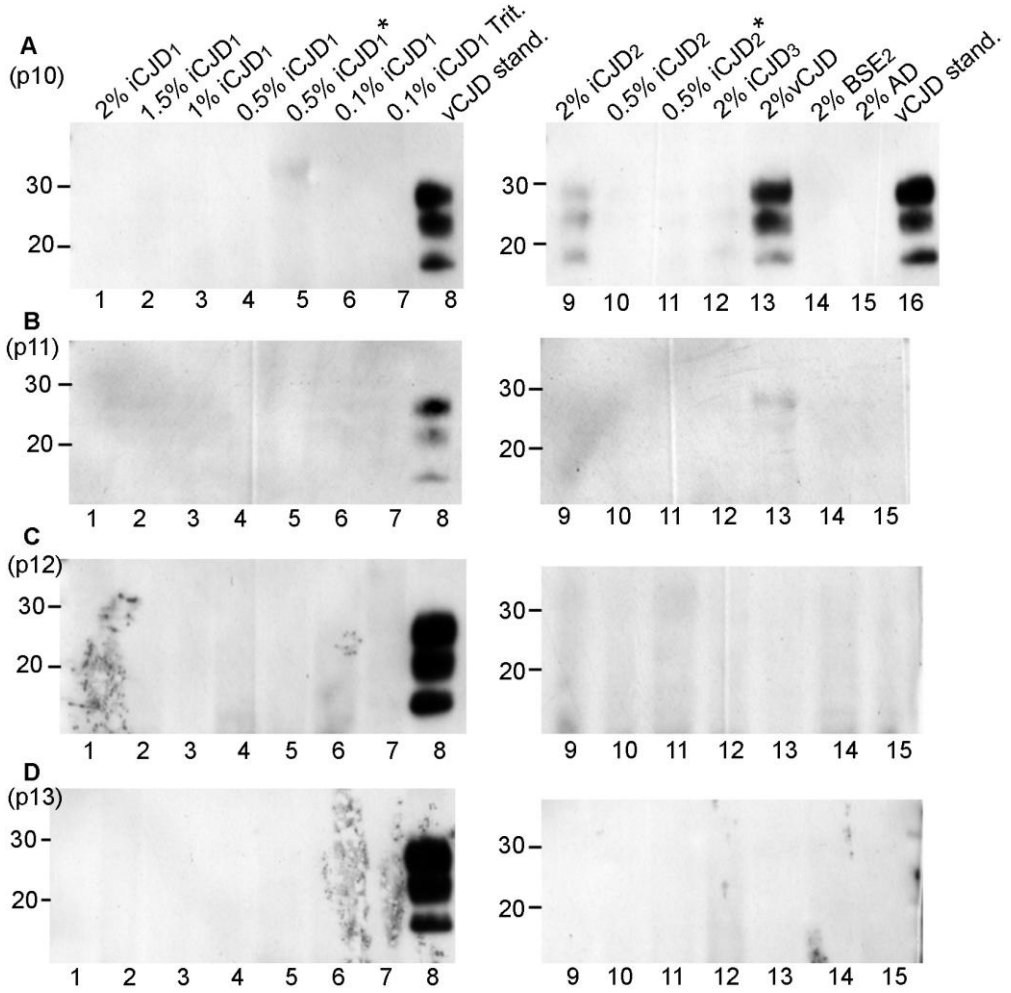


Figure 3.13: HK cells infection studies – fourth set

HK cells, at passage number nine (p9) and 50% confluence, were exposed to various dilutions of ribolysed and sonicated brain homogenates: $iCJD_1$ (lane 1-7), $iCJD_2$ (lane 9-11), $iCJD_3$ (lane 12), vCJD (lane 13), BSE_2 (lane 14) and AD (control) (lane 15), as marked on the figure. In one case the $iCJD_1$ brain homogenate (analysed at lane 7) was only treated with Triton X-100. (*) A pellet of HK cells was gently resuspended in brain spiked medium and cells were plated into cell culture flask. The cells were incubated with the brain spiked medium for 24 hours, then the medium was withdrawn, the cells were washed and given fresh (brain-free) medium. First analysis for the PrP^{res} was carried out 8 days after exposure including one passage of the cultures (A). The experiment was performed in duplicates (as described in section 2.9.2.4), cells in one flask were used for subcultivation, whilst the other flask was used to prepare cell lysate for Western blot analysis (B, C, D). Note that only four time points from eight in total are shown. The cells were harvested and PK digested as described in section 2.6.2.2. To detect PrP^{res}, a primary antibody 3F4 was used. The vCJD 2B standard (lane 8, 16) was used as a control of PrP migration, blotting procedure and as an internal standard to establish a constant film exposure time from time point to time point in order to quantify the data within the linear range of the film. The molecular weight, in kDa, is marked left on each blot.

The loss of the PrP^{res} signal from the cells after subsequent passaging in this assay was obvious, but it is difficult to draw concrete conclusions from the results. They do suggest that clearance of PrP^{res} from the cells exposed to prion diseased brain material does occur, but whether this represents a transient infection or not warranted further investigation.

3.3.3.6 Fifth set of HK cells challenges

In the fifth set of experiments the cell challenge method was modified by using an immobilised brain homogenate. Newly passaged cells were plated onto 0.5% or 1% either Triton X-100 treated (Figure 3.14; A) or ribolysed (Figure 3.14; B) brain homogenates. The brain homogenates were "painted" on the bottom of the cell culture flask, dried overnight and rinsed with PBS prior plating the cells (as described in section 2.9.2.5). The immobilised inoculum was visualised by differential interference contrast microscopy (Figure 3.14). In both cases abundant amounts of brain material was present at the cell culture flask surface. This step was employed as an attempt to obtain a high local concentration of PrP^{Sc} at the basal surface of the cell. The untreated plastic surface of the cell culture flask is shown for comparison (Figure 3.14; C).

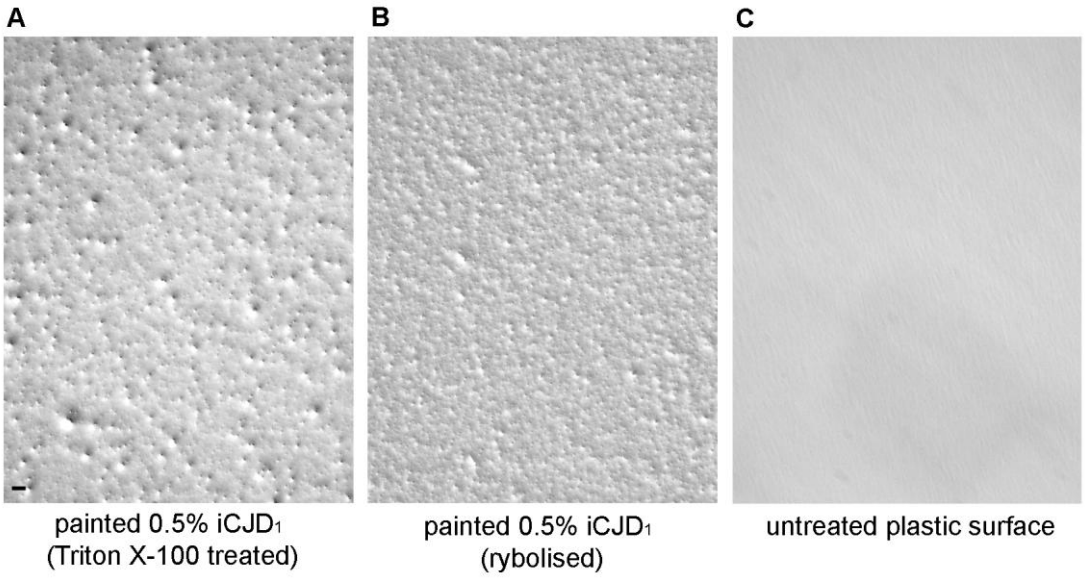


Figure 3.14: Photograph of brain homogenate "painted" on the bottom of the cell culture flask
Immobilised inoculum was observed using a differential interference contrast microscope. PBS dilutions (100 µl) of brain homogenates, either Triton X-100 treated (A) or ribolysed (B) were "painted" on the bottom of the flasks and dried overnight in aseptic conditions. Next day the flasks were rinsed with PBS and HK cells were plated onto the prepared immobilised inoculum. Scale bar, 25 µm.

The incubation time of the HK cell cultures with immobilised inoculum was in this set of experiments prolonged to four days, when the cells were given fresh medium. The experiment was performed in duplicate, thus cells in one flask were used for subcultivation, whilst the other flask was used to prepare cell lysates for Western blot analysis. Cultures in this assay were analysed at six time points in total. These consisted of the first analysis performed after four days incubation with inoculum, followed by one change of medium and then three days of additional incubation (Figure 3.15; a) and five cell passages (split 1:2), each representing an analytical time point (Figure 3.15; b, c, d, e, f). The subsequent passaging was performed to rule out the detection of the remaining PrP^{Sc} from the inoculum and to examine whether PrP^{Sc} replication might increase concomitantly with the loss of the original inoculum. Cells

were harvested using routine method (described in section 2.6.2.2) and the Western blot analysis (described in section 2.7) was performed using anti-PrP monoclonal antibodies 6H4 (Figure 3.15) and 3F4 (Figure 3.16 and 3.17).

A high level of PK resistant PrP^{res} was demonstrated in the first time point analysis of cells exposed to immobilised Triton X-100 treated prion disease inoculum (Figure 3.15; Aa). A marked difference in the amount of cell-associated PrP^{res} in the individual cell lysates was observed (Figure 3.15; Aa, lane 1-5). This was considered to be a dose response effect of PrP^{res} present in the inoculum (Figure 3.9; B). In contrast, cell lysates of cells exposed to the same concentrations, but of immobilised ribolysed inoculum displayed much weaker cell-associated PrP^{res} signal (Figure 3.15; Ba). PrP^{res} successively diminished after first passage analysed six days from the first time point (Figure 3.15; b) and was lost completely after repeated passages during the following 20 days of culturing of the challenged cells (Figure 3.15; b, c, d, e, f). Carry-over of the inoculum was presumably minimised by multiple passaging, and production of newly formed PrP^{res} in the cells was not detected.

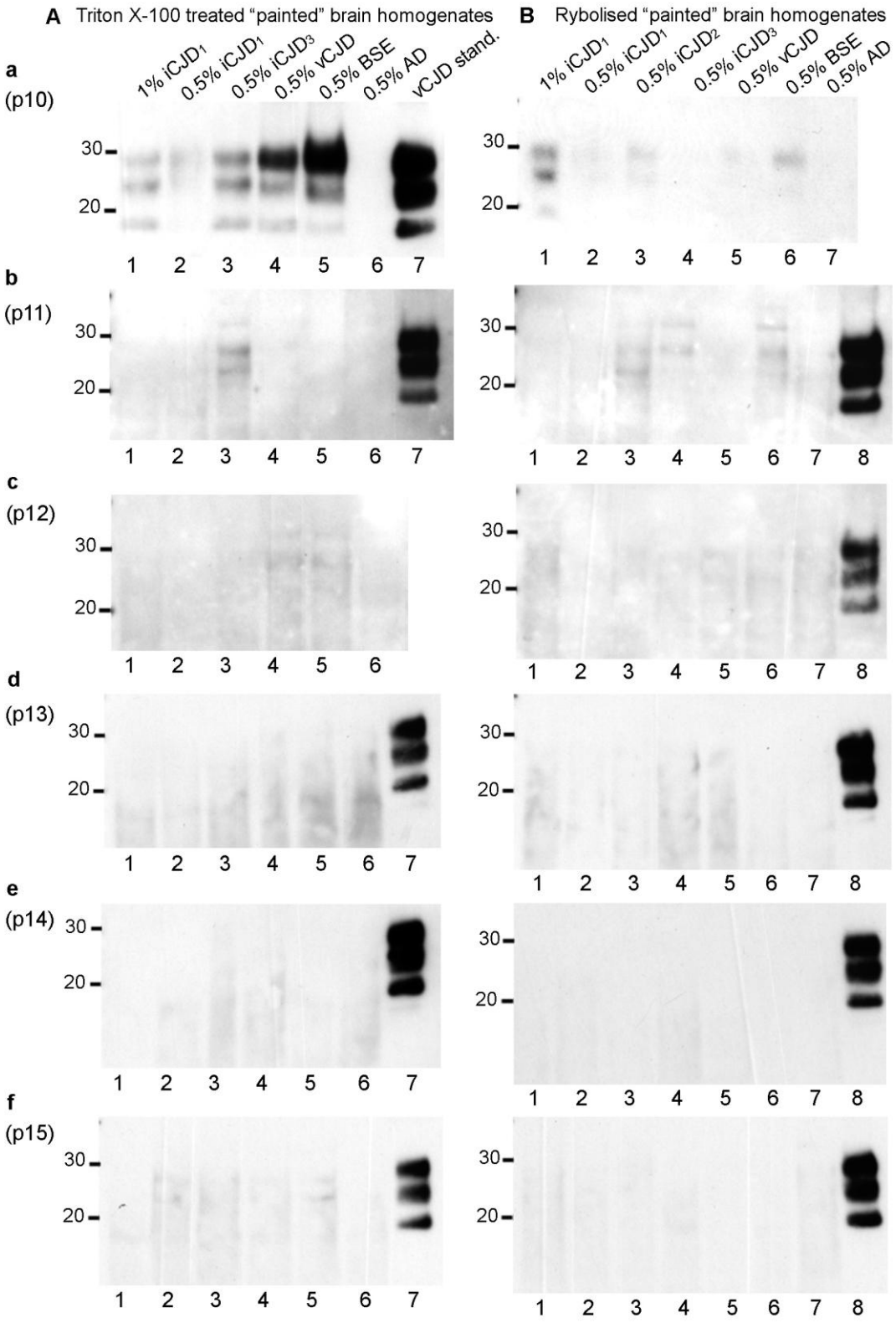


Figure 3.15: HK cells infection studies – fifth set (I.)
HK cells were plated onto immobilised dilutions of iCJD, vCJD, BSE or AD (control) brain homogenates (as marked on the figure), either Triton X-100 treated (A) or ribolysed (B) and incubated for 4 days. The medium was then changed for fresh (unspiked) medium and

cells were cultured for further 3 days, as described in section 2.9.2.5. The cells were then rinsed and harvested for Western blot analysis (a). The experiment was carried out in duplicate. Cells from one flask were harvested for Western blot analysis, whilst cells in the other flask were split into two flasks and were allowed to grow until confluent when harvesting and splitting process was repeated. This process was repeated up to five passages (b-f). Cell samples were PK digested and collected by hard spin (as described in section 2.6.2.2) before analysis by Western blot using the anti-prion primary antibody 6H4 (recognising PrP amino acid residues 144-152) to detect PrP^{res}. The vCJD 2B standard (A, lane 7; B, lane 8) was used as a control of PrP electrophoretic mobility, blotting procedure and as an internal standard to establish a constant film exposure time from time point to time point in order to quantify the data within the linear range of the film. The molecular weight, in kDa, is marked left on each blot.

To further confirm whether the presence of PrP^{res} in early time points originated from the inoculum or represented prion infection, another Western blot analysis was performed using the 3F4 monoclonal antibody (Figure 3.16). The cell lysates of the HK cells exposed to BSE inoculum were investigated, specifically the three cell passage time points p12, p13, p14 representing the 3^{rd}, 4^{th}, 5^{th} time points respectively, were analysed (with 6H4 in Figure 3.15; c, d, e). If the previously detected PrP^{res} originated from newly established infection, it should be detectable by the 3F4 antibody which would only detect human PrP^{res}, but not PrP^{res} of the remaining bovine inoculum. The Western blot of HK cells exposed to BSE immobilised inoculum, either Triton X-100 treated (Figure 3.16; lane 1, 3, 5) or ribolysed (Figure 3.16; lane 2, 4, 6), did not show detectable, newly formed PrP^{res} over three weeks post exposure to the brain spiked medium. No PrP^{res} was observed when cells were exposed to BSE and analysed by 3F4, suggesting that Western blots analysed using 6H4 was actually monitoring the rate at which the inoculum was diluted out from the cell cultures. It was concluded from the analysis that PrP^{res} in HK cells was lost in long term incubation after repeated passages.

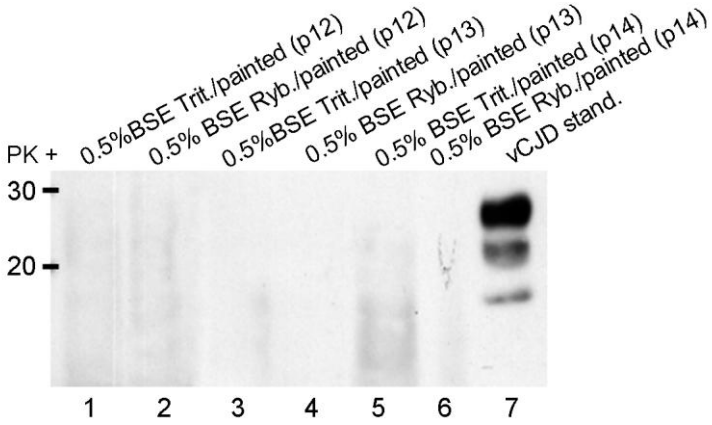


Figure 3.16: HK cells infection studies – fifth set (II.)
HK cells cultured on immobilised "painted" 0.5% BSE brain homogenate (time points 3rd, 4th, 5th), either Triton X-100 treated (lane 1, 3, 5) or ribolysed (lane 2, 4, 6) (as described in section 2.9.2.5 or description of figure 3.15; c, d, e) were also analysed using anti-prion protein 3F4 monoclonal antibody (recognising human, but not bovine, PrP amino acid residues 109-112). Cell samples were routinely processed as described before (section 2.6.2.2). The vCJD 2B standard (lane 7) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left.

3.3.3.7 Sixth set of HK cells challenge with prions

The strategy of the sixth set of HK challenges was based on the preliminary results of reducing of PrP^{res} levels in constitutively infected cell cultures, prion infected mice and delay of PrP^{res} formation caused by certain antibiotics or polyene antimycotics (Mange *et al.*, 2000a; Mange *et al.*, 2000b; Mange *et al.*, 2002; Weissmann and Aguzzi, 2005; Soler *et al.*, 2008). Therefore, in attempt to facilitate PrP^{res} formation in the HK cells exposed to prion material, the challenged cells were cultured in the cell culture medium without a Penicillin/Streptomycin/Amphotericin antibiotics/antimycotics cocktail as Amphotericin B have been reported (Mange *et al.*, 2000b; Weissmann and Aguzzi, 2005; Soler *et al.*, 2008) to interfere with successful PrP^{res} formation in cultured cells (Figure 3.17). To test whether HK cells grown in conditions without antibiotics (ATB) would be more susceptible to

infection with prions, the cultures were exposed to ribolysed and sonicated 0.5% $iCJD_2$ brain spiked medium without ATB for 4 days (Figure 3.17; A). HK cells grown in the culture medium not containing ATB and containing brain homogenate for four days showed no differences in morphology when compared to cells cultured in complete culture medium. The HK cells given fresh (no ATB) containing medium (Figure 3.17; B) and further cultured for three days (Figure 3.17; C) showed some contamination with bacterial or fungal conventional agents in the medium and moderate (Figure 3.17; B and C) to severe (Figure 3.17; D) changes in cell morphology and growth rate could be observed. Despite that, the cultures did not undergo apoptosis and were maintained up to 29 days post challenge with prion material (Figure 3.17; D) when the cultured cells started to show severe changes in morphology and the experiment was terminated. However, staining for presence of bacteria in the culture was not performed to verify this assumption.

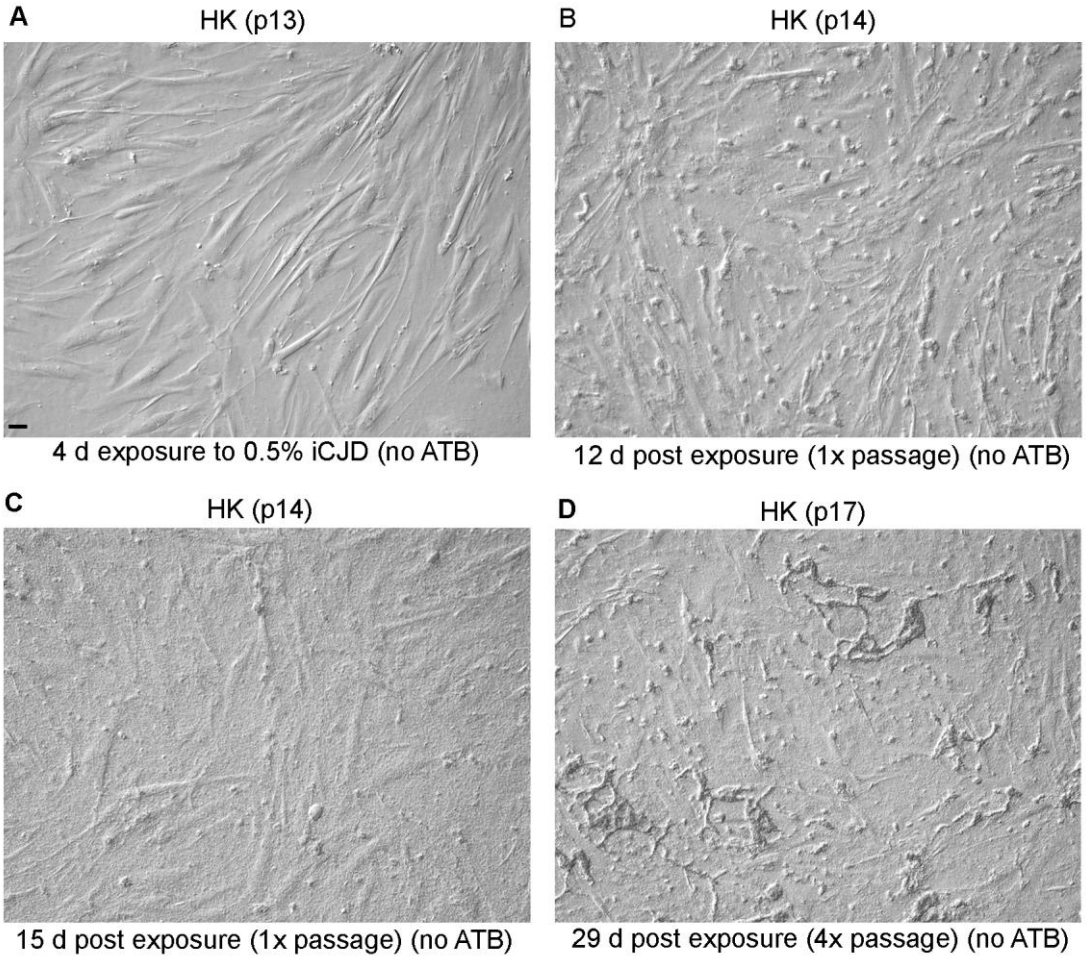


Figure 3.17: Photographs of HK cells cultured in medium without antibiotics after exposure to iCJD brain homogenate - HK cells infection studies – sixth set
Pellets of HK cells were gently resuspended in ribolysed and sonicated 0.5% $iCJD_2$ brain spiked medium with omitted Penicillin/Streptomycin/Amphotericin antibiotics/antimycotics cocktail (ATB) and plated into fresh cell culture flasks. The cells were incubated with the infectious medium for 4 days (A). Then the medium was removed, the cells were washed with PBS and further cultured with fresh medium not containing antibiotics for another 4 days, when the cells were harvested for Western blot analysis. The experiment was carried out in duplicate. Cells from one flask were harvested for Western blot analysis and the cells from the other flask were split into two flasks and cultured until confluent (B, C) when harvesting and splitting process was repeated (as described in section 2.9.2.6). This process was repeated up to four passages from cells being exposed to brain material, when the experiment was terminated (D). Challenged HK cells given fresh medium not containing antibiotics (B) and maintained in this medium for 3 days is shown in (C). The cells were observed by a differential interference contrast microscope. Scale bar, 100 µm.

HK cells were exposed to the ribolysed and sonicated 0.5% $iCJD_2$ brain spiked medium when freshly passaged and were incubated in the infectious medium for four days (three days and one day with additional 2 ml of fresh medium, as described in section 2.9.2.6). The medium was then withdrawn, cells were rinsed with PBS, given fresh (brain-free) culture medium not containing ATB and further maintained for four days, when the culture was harvested for PrP^{res} detection by Western blot using the 6H4 antibody (Figure 3.18; lane 1). The experiment was carried out in duplicate. Cells were maintained for an additional three weeks, consisting of four passages representing time points of cell lysates analysed for PrP^{res} (Figure 3.18; lane 2-5). The PrP^{res} signal seemed to slowly diminish from the challenged cells by repeated passaging. Interestingly, the process of clearance of the PrP^{res} signal seemed to be slower when compared with data observed in previous experiments.

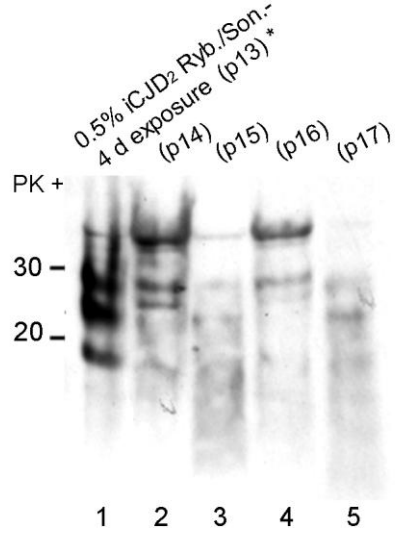


Figure 3.18: HK cells infection studies – sixth set
A pellet of HK cells was gently resuspended in ribolysed and sonicated $iCJD_2$ brain homogenate diluted in a cell culture medium without Penicillin/Streptomycin/Amphotericin antibiotics (ATB) to 0.5% brain suspension. The cells were subsequently plated into fresh cell culture flasks and incubated with the infectious medium for 4 days. The medium was then removed, cells were washed and maintained for additional 4 days in a fresh medium (free of ATB), when they were harvested for Western blot analysis (lane 1). Experiment was carried out in duplicate and as described in section 2.9.2.6. One flask of cells was harvested for Western blot analysis and the other flask was split into two flasks and cultured. This

procedure was repeated every ~ 5 days (lane 2, 3, 4, 5). The experiment was terminated after four passages (p17, lane 5). At each harvesting time point, the cells were extensively washed, lysed with extraction buffer, digested with 50 μg/ml PK for 60 minutes at 37 °C and then collected by centrifugation at 14,000 rpm for 60 minutes as described in section 2.6.2.2. Western blot analysis was routinely carried out using 6H4 primary antibody (as described in section 2.7). The molecular weight, in kDa, is marked left.

3.3.3.8 Seventh set of HK cell challenges

The seventh set of challenges investigated the effect of changes in cell culture conditions on cell susceptibility to infection with prions. This attempt was designed to examine whether cells grown in poor nutritional conditions (serum starvation - low Foetal Calf Serum content, 0.5% FCS) would be more sensitive to infection with prion than those grown in complete cell culture medium containing normal level of Foetal Calf serum (10% FCS).

The HK cells were either maintained in a normal medium containing 10% FCS (Figure 3.19; A) or a nutrition-low medium containing 0.5% FCS (Figure 3.19; B) for 3 days, prior exposure to sonicated 1% $iCJD_1$ brain spiked medium. The brain homogenate was diluted in a medium either containing 10% or 0.5% FCS, according to the type of medium, the cells were cultured in prior to the exposure. The cells were incubated with the brain spiked medium for 48 hours. Homogeneity in HK cell morphology could be observed in cell populations maintained under both culture conditions, although as anticipated cells grown in poor nutritional conditions (Figure 3.19; B and D) were observed to have a slower growth during the period of poor nutritional conditions, when compared to cells grown in normal conditions (Figure 3.19; A and C).

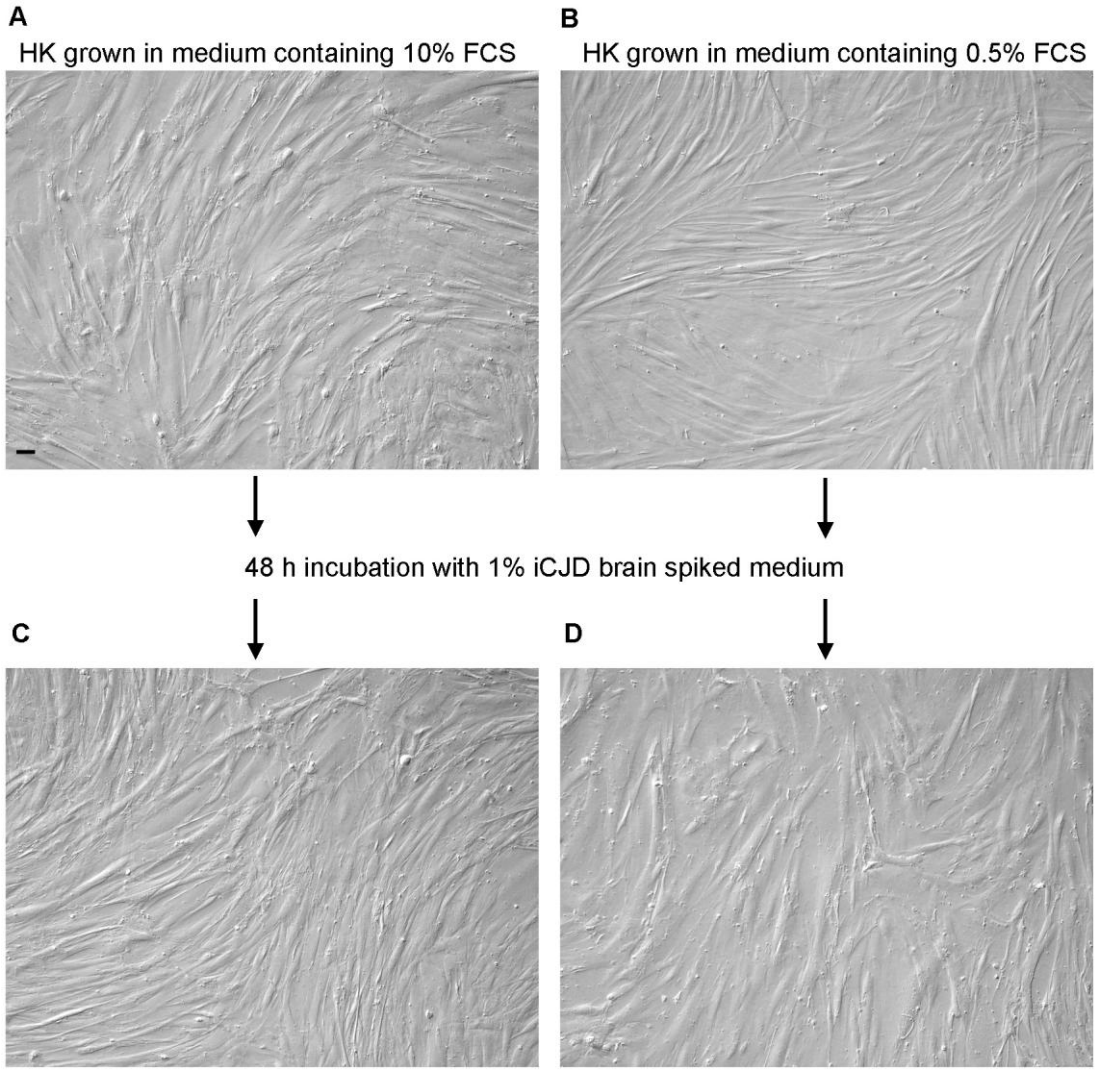


Figure 3.19: Photomicrograph of HK cells cultured in normal or low nutrient medium
Pellets of HK cells were resuspended in medium containing normal level of foetal calf serum (FCS) – 10% and cultured for 3 days. Then one group of the cells continued culturing in the normal medium (A) and the second group was washed with PBS and cultured in poor nutritional conditions – medium containing 0.5% FCS (B) for 3 days. The cells were then exposed to 1% $iCJD_1$ brain homogenate diluted either in the medium containing 10% FSC (C) or 0.5% FCS (D) for 48 hours. The cells were exposed to dilution type corresponding to the type of medium they were cultured prior challenge with brain spiked medium. The cells were observed using a differential interference contrast microscope. Scale bar, 100 µm.

HK cells were analysed for PrP^{res} by Western blot at 24 (Figure 3.20; Aa and Ba, lane 1) and 48 hours (Figure 3.20; Aa and Ba, lane 2) of exposure to the brain spiked medium. At this time point, a strong signal for cell-associated PrP^{res} was observed in

cells maintained in normal nutritional conditions (Figure 3.20; Aa, lane 1 and 2). Interestingly, the signal of cell-associated PrP^{res} in cell lysates from cells cultured in poor nutritional conditions appeared weaker (Figure 3.20; Ba, lane 1 and 2) when compared to cells grown in normal conditions. This may be the result of a lower rate of cell division, and therefore fewer cells being present at the time of harvesting when compared to normal cell growth conditions.

After 48 hours the brain spiked medium was removed, cells were rinsed with PBS and given fresh (brain-free) cell culture medium containing either 10% or 0.5% FCS. The cultures were allowed to grown for a further three days.

The experiment was carried out in duplicate. One flask of cells was then harvested for Western blot analysis (Figure 3.20; Aa and Ba, lane 3) at a three day recovery time point. At that point, all challenged cells were given complete cell culture medium (containing 10% FCS). Medium changes took places every three days. Analysis for PrP^{res} was then carried out on the 6^{th} (Figure 3.20; Aa and Ba; lane 4) and the 9^{th} (Figure 3.20; Aa and Ba, lane 5) days of culturing with complete and brain-free medium. Another analysis for PrP^{res} was performed after the first passage on the 12^{th} (Figure 3.20; Aa and Ba, lane 6), 24^{th} (Figure 3.20; Aa and Ba, lane 7) and in the second passage on the 40^{th} day of recovery (Figure 3.20; Aa and Ba, lane 8). In both groups, 10% FCS and 0.5% FSC, the previously observed cell-associated PrP^{res} signal was seen to slowly diminish. In this assay, the medium cultured with the cells was also analysed for PrP^{res}, to determine whether the previously cell-associated PrP^{res} was then shed back to the culture medium, or whether it might be degraded by the cell's proteolytic machinery. A gradual loss of PrP^{res} from the medium was observed (Figure 3.20; Ab and Bb, lane 1 and 2) concomitant with its appearance as

cell-associated PrP^{res} (Figure 3.20; Aa and Ba, lane 1 and 2). However, the analysis of the medium at recovery time points did not show any detectable PrP^{res} shed back to the medium (Figure 3.20; Ab and Bb, lane 3-8). Neither could it be detected as cell associated in cell lysates at these later time points (Figure 3.20; Aa and Ba, lane 5-8).

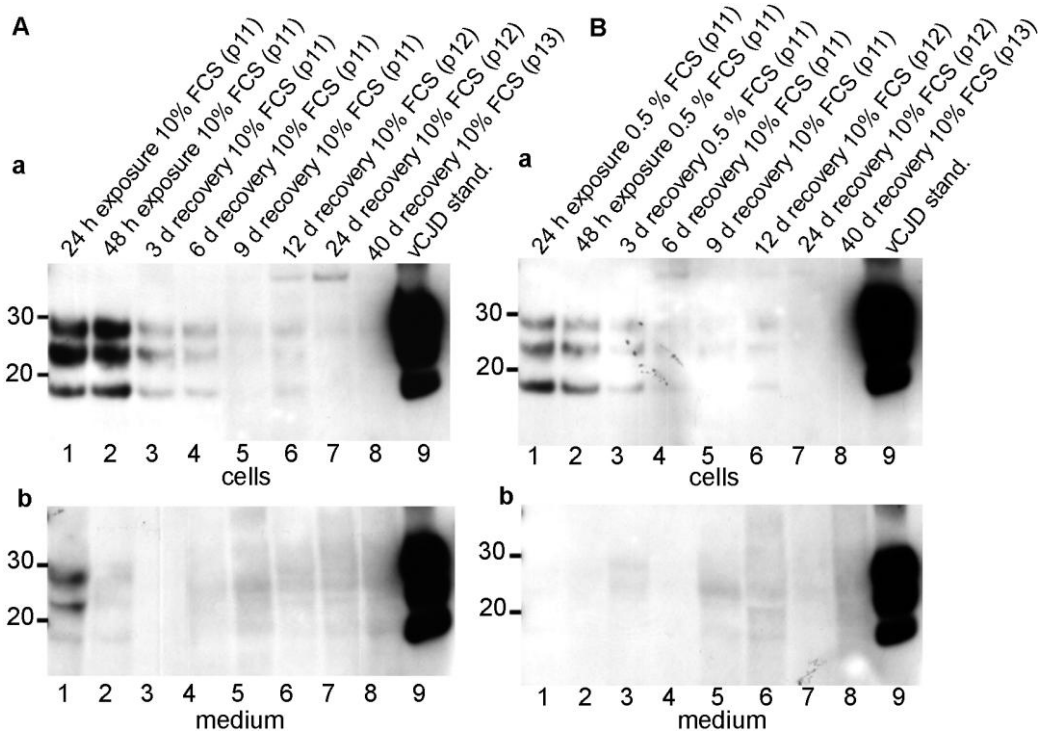


Figure 3.20: Seventh set of HK cells infection (cells cultured in normal or nutrient-low conditioned medium)

HK cells were either maintained in a normal medium (containing 10% FCS) (A) or a nutrition-low medium (0.5% FCS) (B) for 3 days prior exposure to the 1% $iCJD_1$ brain spiked medium for 48 hours. This brain spiked medium contained either 10% or 0.5% FCS, according to the type of medium cells were cultured in prior exposure. Cells were harvested for Western blot analysis at 24 (lane 1) and 48 hours (lane 2) of continuous exposure. The medium was then removed, the cells were washed and further cultured with a fresh culture medium (containing either 10% or 0.5% FCS) for 3 days and then the cells were harvested for Western blot analysis (lane 3). At this point, both groups of cells were given a fresh medium containing 10% FCS. Analysis for PrP^{res} was carried out on, the 6^{th} (lane 4), the 9^{th} (lane 5) days of culturing with complete and brain-free medium, also after 1^{st} passage and on the 12^{th} (lane 6), the 24^{th} (lane 7) and the 2^{nd} passage on the 40^{th} day of recovery (lane 8). At each harvesting time point, the medium cultivated with cells was retained (b) and the cells (a) were extensively washed and lysed with extraction buffer. Both (cells and medium) were then digested with PK and then collected by centrifugation as described in sections 2.6.2.2 and 2.6.3. Western blot analysis was routinely carried out using 3F4 primary antibody (as described in section 2.7). The vCJD 2B standard (lane 9) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.3.3.9 Evaluation of PrP^{res} tendency to bind to the plastic surfaces

A control experiment investigating the potential of PrP^{res} binding to plastic was also carried out. This was to determine whether there was any potential of PrP^{res} in these experiments to strongly bind to the plastic surface of the cell culture flasks, which could be wrongly considered as cell associated PrP^{res} when cells were exposed to infection brain material and harvested for Western blot analysis. Cell culture flasks (not containing cells) were incubated with either 0.15% ribolysed, sonicated and filtered (ryb/son/filt) $iCJD_1$ or 0.05% Triton X-100 treated brain spiked medium for 28 hours. The input of ryb/son/filt 0.15% iCJD is shown in Figure 3.21; lane 1. The input of 0.05% Triton X-100 treated iCJD is shown in Figure 3.21; lane 5 (note that this sample was not PK treated before Western blot analysis). The flasks were washed with PBS and then scraped with extraction buffer, like in all previous experiments, as they would contained cell culture (as described in section 2.6.2.2) and the suspensions were retained. The suspensions were then PK treated and analysed by Western blot using the 6H4 antibody. No PrP^{res} was detected after 28 hours of incubation of the iCJD brain spiked medium (both ryb/son/filt 0.15% or 0.05% Triton X-100 treated) on the surface of the plastic cell culture flasks (Figure 3.21; lane 2, 6, respectively). The medium incubated for 28 hours in the flasks was also retained, PK treated and processed for Western blot analysis (as described in section 2.6.3). PrP^{res} was detected in the medium (Figure 3.21; lane 3 and 7). The 4^{th} PBS rinse of the cell culture flasks was also retained and analysed (Figure 3.21; lane 4 and 8). No PrP^{res} could be detected in the 4^{th} PBS rinse, which confirmed that four PBS washes were sufficient enough to wash any unbound prion material from the cell culture surface in the infection assays.

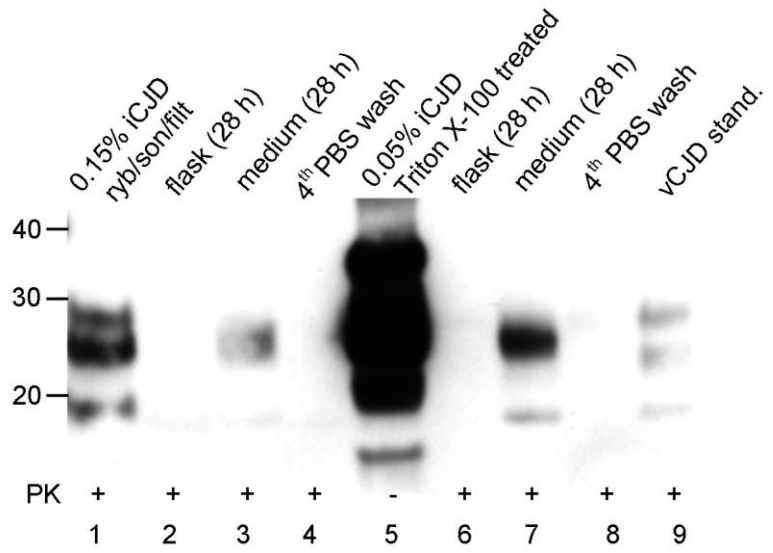


Figure 3.21: Evaluation of PrP^{res} tendency to bind to the plastic surfaces
Two cell culture T25 flasks were incubated with the brain spiked medium: 0.15% ribolysed and sonicated $iCJD_1$ (lane 1-4) and 0.05% Triton X-100 treated $iCJD_1$ (lane 5-8) for 28 hours. The medium was then withdrawn and transferred in tubes, flasks were rinsed four times with PBS and the 4th wash was retained. Volume of 100 µl extraction buffer was added to the flasks and scraped into tubes. All samples were then PK digested and then collected by centrifugation as described in sections 2.6.2.2 and 2.6.3. Western blot analysis was carried out using 6H4 primary antibody (as described in sections 2.7). The vCJD 2B standard (lane 9) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left.

3.3.4 Summary

- Despite the wide variety of inoculum and exposure protocols employed, these data lead to the conclusion that HK cells are largely resistant to infection with human and bovine prions or that they are not able to sustain prion infection.
- Even when human adapted prions (iatrogenic CJD) of the appropriate *PRNP* codon 129 genotype were used, no evidence was found for a stable infection of the HK cells.
- The most likely explanation for the PrP^{res} signal observed in Western blots of the cell lysates from cells exposed to prion disease brain material at early time points is that it represents residual inoculum, which was then lost during longer term culturing and subsequent passages and analysis using the 3F4 and 6H4 antibody in cells exposed to BSE is consistent with this explanation.
- The mechanism by which cell-associated PrP^{Sc} is lost during this process is not known, but is of interest and was investigated later in this project.
- Despite the findings in here, it cannot be completely discounted that some HK cells may suffer a transitory or perhaps fatal prion infection within the exposed cultures.

3.4 WESTERN BLOT ANALYSIS OF HK CELLS FOR PrP^{Sc} UPTAKE AFTER ACUTE EXPOSURE TO PRION DISEASED BRAIN MATERIAL

3.4.1 Objectives

To attempt to quantitate HK cell uptake of exogenous PrP^{Sc} after an acute exposure to prion diseased brain material.

3.4.2 Rationale

The previous experiments (section 3.3) showed that after exposure to infectious prion brain material, regardless of the prion agent, the HK cells were not able to support PrP^{Sc} replication and propagate prion infection *in vitro*. It was therefore of interest to examine the interactions between PrP^{Sc} and human FDC-like HK cells during the acute stage of exposure to infectious prions and also to investigate whether there was any evidence of an initially successful, although transient, infection.

3.4.3 Experimental results

To determine the rate the exogenous PrP^{Sc} is taken up by HK cells, the infectious brain homogenate was diluted in medium and incubated with the cells for a range of times (as described in section 2.10). At each time point the medium incubated with the cells was withdrawn (and retained for analysis), cells were extensively washed with PBS to remove any loosly bound PrP^{Sc} (the 1^{st} and 4^{th} PBS wash were retained for analysis) and the cells were solubilised in detergent-containing lysis buffer. Then the samples were PK digested and insoluble material pelleted by hard spin as described in sections 2.6.2.2 and 2.6.3 and the pellet was assayed for PrP^{Sc} by

Western blot as described in section 2.7. Unchallenged HK cells were used as a control.

The PrP^{Sc} uptake by HK cells analysed by WB is representative of eight independent, but non-identical experiments (in the case of cells exposure to iCJD) and three independent, non-identical experiments in the case of cells exposure to vCJD. The differences involved the time points assayed (not all data are shown).

3.4.3.1 Uptake of PrP^{Sc} by HK cells after incubation with iCJD brain homogenate (time points: 30 minutes – 48 hours of continuous exposure)

Firstly, to investigate the rate at which the PrP^{Sc} was taken up, the cells were exposed to 0.05% iCJD brain homogenate and analysed for presence of any cell associated PrP^{Sc} as described in section 2.10.1 and summarised in Figure 3.22.

The results showed that PrP^{Sc} uptake is very rapid. Cell associated PrP^{Sc} was faintly detectable in cell lysates at 1 hour (A, lane 4) and readily detectable after 2 hours of incubation (A, lane 5). The intensity of cell-associated PrP^{Sc} signal increased with time of exposure (A, lane 4-9), while the PrP^{Sc} signal started to reduce in the medium at 6 hours of incubation (B, lane 7) and became undetectable in the iCJD brain spiked medium after 24 – 48 hours of incubation (B, lane 8, 9). A faint PrP^{Sc} signal was present in the 1st PBS wash of exposed cells (C), but PrP^{Sc} could not be detected in 4th PBS wash (D), indicating that any residual unbound PrP^{Sc} was effectively removed from the cell monolayer by four rinses of PBS and that the PrP^{Sc} signal detected in cell lysate (A) was firmly cell-associated.

The remaining PrP^{Sc} from the input could be detected in the medium analysed from this time point only faintly (B, lane 8), suggesting that the remainder of the PrP^{Sc}

from the input was most likely poorly cell associated (bound to the cell surface) and was effectively washed off by the four PBS rinses foregoing cell harvesting.

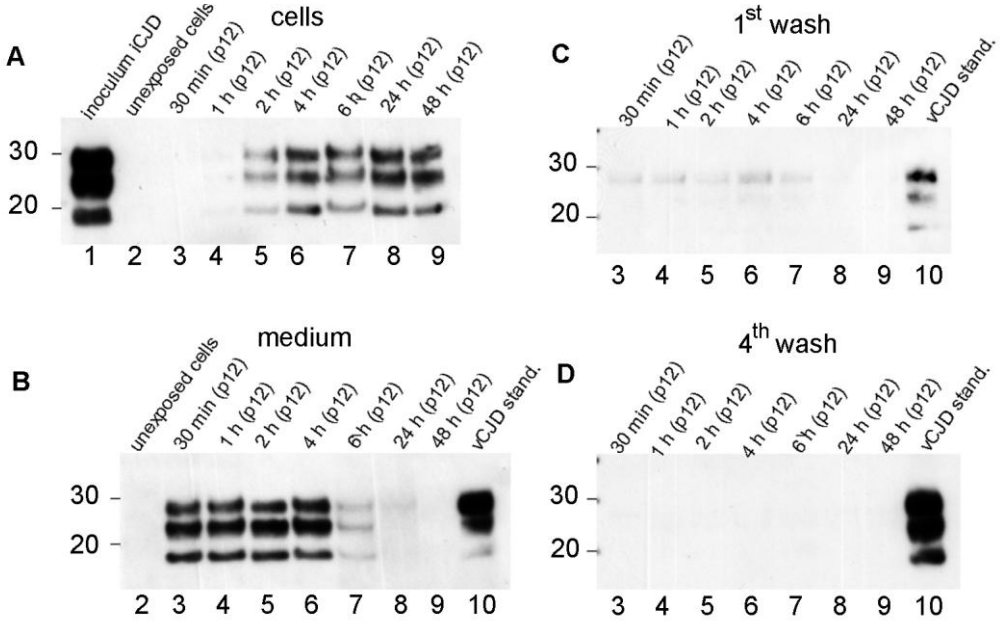


Figure 3.22: Representative Western blot analysis of cell associated PrP^{Sc} after HK cells incubation with iCJD brain homogenate (time points: 30 minutes – 48 hours of continuous exposure)

HK cells continuously incubated with a 0.05% $iCJD_1$ brain spiked medium were harvested for Western blot analysis at various time points (as described in section 2.10.1 and marked on the figure). Briefly, cell samples (A) were extensively washed, lysed with extraction buffer, digested with 50 μg/ml PK for 60 minutes at 37 °C and then collected by centrifugation at 14,000 rpm for 60 minutes. Medium - the cells were incubated with (B), 1st (C) and 4th (D) PBS wash of each time point were also retained, PK treated and collected by centrifugation. (A, lane 1) corresponds to the exact iCJD input of each time point. Pellets of all samples were resuspended in an appropriate volume of sample buffer and subjected to Western blot analysis using mAb 3F4. The vCJD 2B standard (lane 10) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.4.3.2 Uptake of PrP^{Sc} by HK cells after incubation with vCJD brain homogenate (time points: 30 minutes – 48 hours of continuous exposure)

vCJD was also used to investigate potential strain-specific differences in PrP^{Sc} uptake by HK cells after acute exposure to prion diseased medium. The HK cells were exposed to 0.02% vCJD brain homogenate as described in section 2.10.1 and analysed for the rate of PrP^{Sc} uptake from this brain homogenate is shown in Figure 3.23. Preparation of the samples was the same as described in the previous experiment.

The result of PrP^{Sc} uptake from vCJD spiked medium was qualitatively similar to the previous observations using iCJD. (A, lane 1) corresponds to the exact vCJD input for each time point. The signal of cell-associated PrP^{Sc} was already detectable at 1 hour of exposure (A, lane 4) and the amount of cell-associated PrP^{Sc} increased with time of exposure (A, lane 4-9). In addition, the PrP^{Sc} signal was lost from the brain spiked medium after 48 hours of incubation (B, lane 9). PrP^{Sc} was just detectable in the pellet from high speed spin of the 1st PBS wash (C), but could not be detected in the 4th PBS wash (D), indicating that any residual unbound PrP^{Sc} was effectively removed by four PBS washes prior the cell lysis.

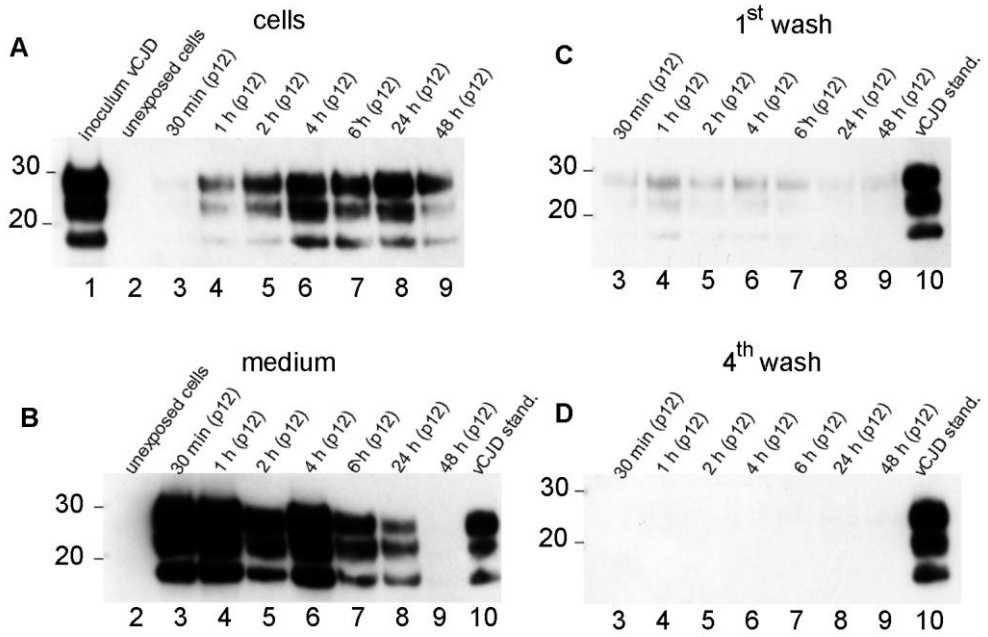


Figure 3.23: Western blot analysis of cell associated PrP^{Sc} after HK cells incubation with vCJD brain homogenate (time points: 30 minutes – 48 hours of continuous exposure)

HK cells continuously incubated with a 0.02% vCJD brain spiked medium were harvested for Western blot analysis at various time points (as described in section 2.10.1 and marked on the figure). The cell samples (A) were extensively washed, lysed with extraction buffer, digested with 50 µg/ml PK for 60 minutes at 37 °C and then collected by centrifugation at 14,000 rpm for 60 minutes as described in section 2.6.2.2. (A, lane 1) corresponds to the exact vCJD input for each time point. The medium (B), 1st (C) and 4th (D) PBS wash of each time point were also retained, PK treated and collected by centrifugation as described in section 2.6.3. The pellets were resuspended in an appropriate volume of sample buffer and subjected to Western blot analysis using mAb 3F4. The vCJD 2B standard (lane 10) was used as a control of PrP migration and blotting procedure. The molecular masses are shown in kDa, left on each blot.

3.4.3.3 Uptake of PrP^{Sc} by freshly plated HK cells and incubated with iCJD brain homogenate (time points: 1 – 48 hours of continuous exposure, 1st, 2nd and 3rd cell passage post exposure – 51 days)

The next experiment was designed to investigate whether passaging of the cells delays the PrP^{Sc} uptake into the freshly plated cells. The experiment was designed as described in section 2.10.2. Cell pellets of the HK cells were gently resuspended in 0.05% iCJD brain spiked medium. Then the cells were plated into fresh cell culture

flasks and continuously incubated with the infectious medium up to 48 hours or until harvested for analysis for cell associated PrP^{Sc} at a range of early time points as shown in Figure 3.24. The samples analysed by Western blot were the same as in previous studies: cell lysate, medium incubated with the cells, 1st and 4th PBS wash. A cell associated PrP^{Sc} signal was detected at 6 hours of exposure (A, lane 4). Then the infectious medium was withdrawn, cells were washed, split at a ratio of 1:2 by passaging and allowed to grow in fresh (unspiked) complete cell culture medium until 51 days from exposure, which consisted of three cell passages analysed at each time point (A, lane 7-9). The amount of cell-associated PrP^{Sc} increased with time of exposure (A, lane 2-6) and the PrP^{Sc} signal became barely detectable in the brain spiked medium after 48 hours of incubation with HK cells (B, lane 6). However, the cell associated PrP^{Sc} could not be detected at any time points following the first cell passage and culturing of the cells for 18 days (A, lane 7). Neither was it detectable after the next two passages (A, lane 8, 9). PrP^{Sc} signal was not detectable in the 1st (C) and 4th (D) PBS wash either.

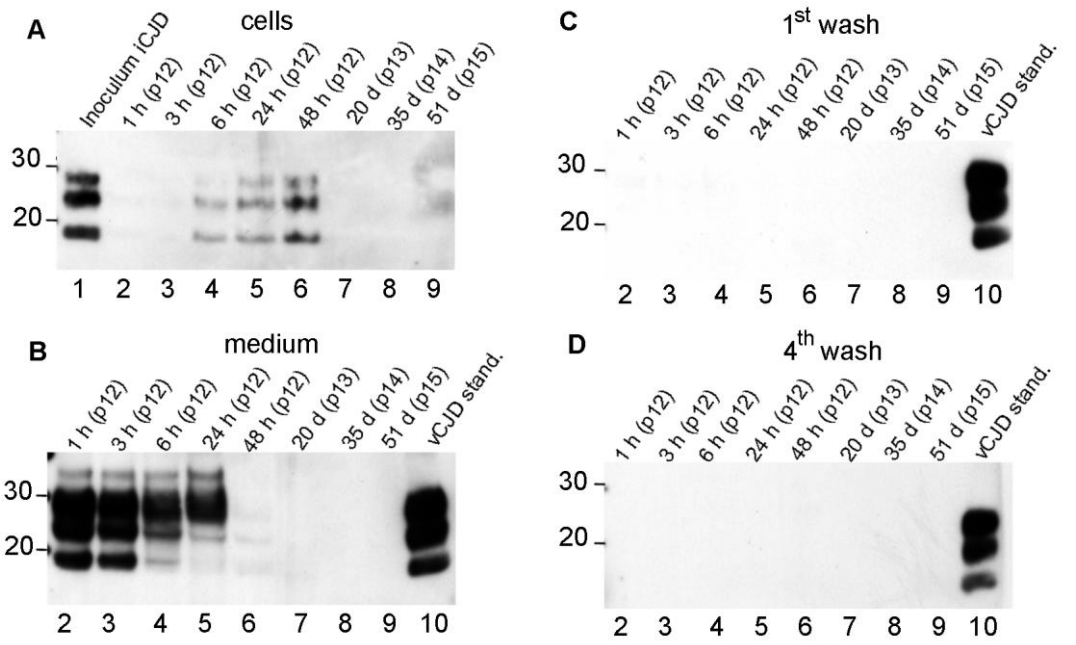


Figure 3.24: Western blot analysis of cell associated PrP^{Sc} after HK cells incubation with iCJD brain homogenate (time points: 1 – 48 hours of continuous exposure, 1st, 2nd and 3rd cell passage post exposure – 51 days)

Pellets of HK cells (after centrifugation step in cell culture passaging procedure) were resuspended in a complete cell culture medium containing $iCJD_1$ brain homogenate at concentration 0.05% (A, lane 1 – input) and incubated at 37 °C. Cells were then harvested at various time points (as described in section 2.10.2 and marked on the figure). Harvested cells (A), medium (B), 1st (C) and 4th (D) PBS wash of each time point were retained, PK treated and collected by centrifugation at 14,000 rpm for 60 minutes. The pellets were resuspended in an appropriate volume of sample buffer and subjected to Western blot analysis using mAb 3F4. The cell passages analysed at days from exposure to brain spiked medium are marked as p13 (lane 7), p14 (lane 8), and p15 (lane 9). The vCJD 2B standard (lane 10) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.4.3.4 Analysis of PrP^{Sc} uptake after HK cells were continuously incubated with iCJD brain homogenate for 24 hours followed by a 24 hours recovery and culturing of the cells until senescent

It was of interest to re-test whether the iCJD exposed HK cells might be replicating PrP^{Sc} at levels undetectable by Western blot at earlier time points post-exposure and culturing, and whether the PrP^{Sc} signal would appear in the cells at detectable levels after long-term culturing. Accordingly, the cells were cultured and analysed until becoming senescent at 177 days post-exposure to iCJD brain spiked medium. The experiment was designed as described in section 2.10.3 and the HK cells were analysed for cell-associated PrP^{Sc} as shown in Figure 3.25. The cells were continuously exposed to 0.05% $iCJD_1$ brain spiked medium for 24 hours. The intensity of cell-associated PrP^{Sc} signal increased with time of exposure (A, lane 2-5), while the PrP^{Sc} signal was barely detectable in the medium after 24 hours of incubation (B, lane 5). Then the infectious medium was removed, and the cells were washed and cultured in fresh (unspiked) complete culture medium for 24 hours. PrP^{Sc} was detected in the cell lysate after the 24 hours recovery (A, lane 6).

Interestingly, the signal at this time point appeared to be more intense than in the cell lysate of cells analysed after the 24 hours exposure (A, lane 5).

No PrP^{Sc} could be detected in medium cultured with the cells after 24 of hours recovery time (B, lane 6). Analysis of the 1^{st} and 4^{th} PBS washes (C, D) showed no detectable PrP^{Sc}.

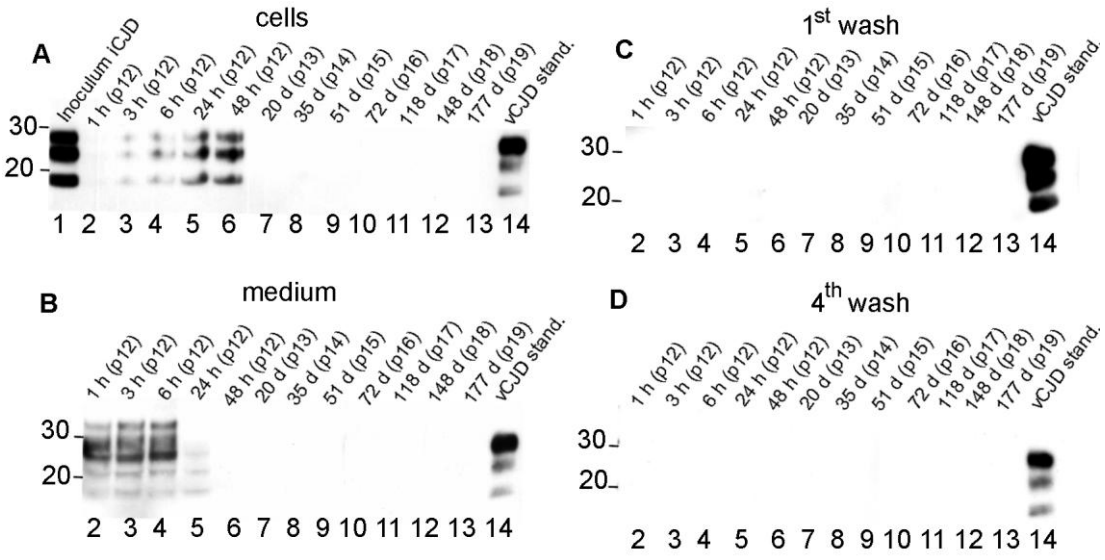


Figure 3.25: Western blot analysis of cell associated PrP^{Sc} after HK cells continuous incubation with iCJD brain homogenate for 24 hours followed by a 24 hours recovery and culturing of the cells until senescent

HK cells continuously incubated with the 0.05% $iCJD_1$ brain spiked medium (A, lane 1 – input) were harvested for Western blot analysis of any cell associated PrP^{Sc} at 1, 3, 6, and 24 hours of incubation (lane 2, 3, 4, and 5 respectively). The cells were then extensively washed and cultured with fresh medium for another 24 hours - recovery (48 hours time point, lane 6). Subsequently the cells were split at 1:2 ratio and continuously cultured until senescent (p19, lane 13) preserving each passage as a time point for Western blot analysis (lane 7-13). Scheme of the experiment was shown in 2.10.3. Harvested cells (A), medium (B), 1st (C) and 4th (D) PBS wash of each time point were treated as described previously in sections 2.6.2.2 and 2.6.3 and subjected to Western blot analysis using mAb 3F4. The vCJD 2B standard (lane 14) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.4.3.5 Detailed course of the uptake and clearance of exogenous PrP^{Sc} by HK cells exposed to iCJD

To determine the course of PrP^{Sc} uptake by HK cells exposed to prion diseased medium and its subsequent loss from the cells after the medium was withdrawn, a further experiment was designed as shown in Figure 3.26. Based on previous observations, the time points in this assay were considered sufficiently frequent enough to allow the course to be ascertained. The cells were firstly continuously exposed to a 0.05% iCJD brain-spiked medium for 48 hours (A, lane 2-5). The

medium was then removed, cells were washed and allowed to grow in fresh (prion disease brain-free) medium for up to 120 hours of the recovery (with media changes every 24 hours) (A, lane 6-10). The analysis was terminated after the 1st passage of the cells (A, lane 11).

The observations indicated a gradual PrP^{Sc} uptake from the brain-spiked media by the exposed cells (A, lane 2-5). Interestingly, the cell-associated PrP^{Sc} signal was more intense in cell lysate at the 1, 3, and 24 hours recovery time points (A, lane 6-8) when the cells were transferred into fresh medium (when compared to 24-48 hours of continuous exposure; A, lane 4, 5), but the signal gradually diminished with further recovery time (A, lane 9-10). After the 1st passage, the cell-associated PrP^{Sc} could not be detected in the cell lysate any more (A, lane 11). The medium incubated with the cells (B), 1st (C) and 4th (D) PBS washes of each time point were also retained and analysed.

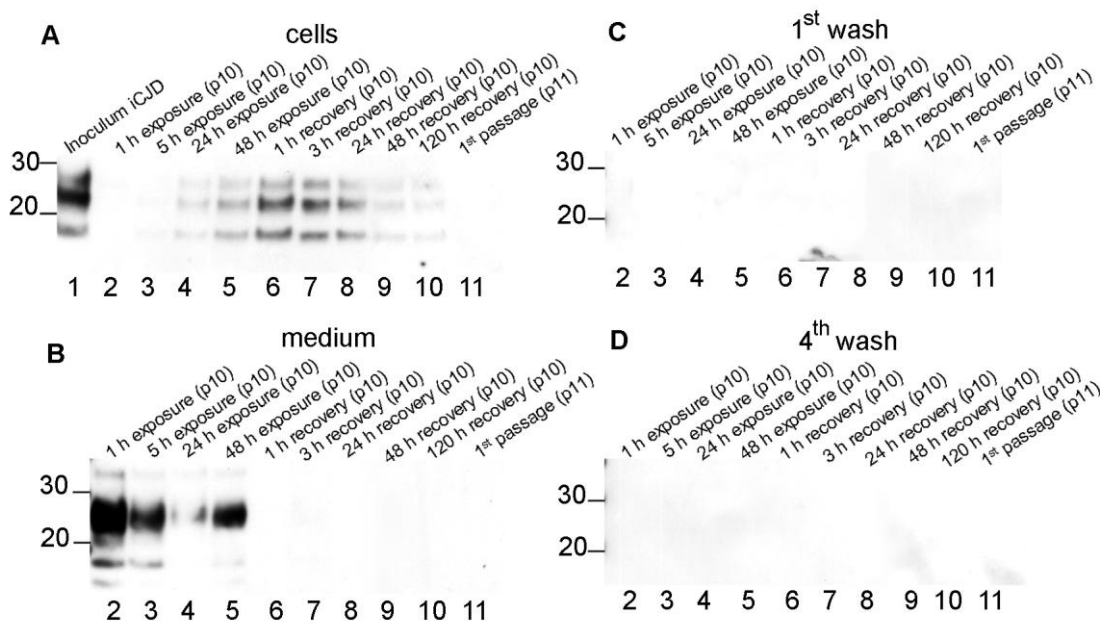


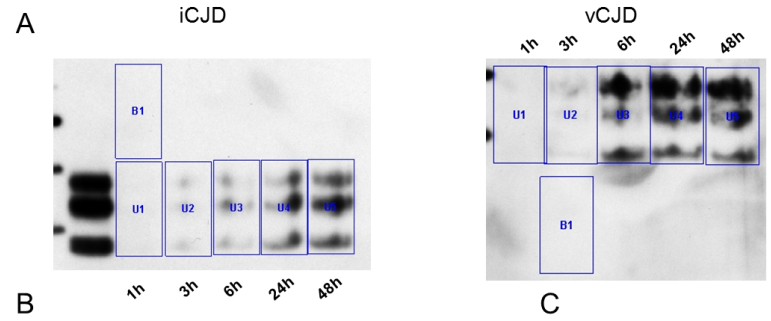
Figure 3.26: Representative Western blot analysis of cell associated PrP^{Sc} after HK cells continuous incubation with iCJD brain homogenate for 48 hours and subsequent culturing in fresh medium for 120 hours - recovery

HK cells were continuously exposed to a 0.05% $iCJD_1$ brain spiked medium (A, lane 1 – input) for 48 hours and analysed for cell associated PrP^{Sc} at 1, 5, 24, and 48 hours (lane 2, 3, 4, and 5 respectively). The cells were then extensively washed and allowed to grow in fresh complete medium for 120 hours with three medium changes - recovery. Cells were analysed at 1, 3, 24, 48, and 120 hours of the recovery time (lane 6, 7, 8, 9, and 10 respectively). Subsequently the cells were split at ratio 1:2 and grow until confluent when analysed as another time point (lane 11 – 1st passage). Harvested cells (A), medium (B), 1st (C) and 4th (D) PBS wash of each time point were treated as described previously in sections 2.6.2.2 and 2.6.3 and subjected to Western blot analysis using 6H4 monoclonal antibody. The molecular weight, in kDa, is marked left on each blot.

3.4.3.6 Densitometric assessment of the time-course of PrP^{Sc} uptake by HK cells after incubation with iCJD or vCJD brain homogenate

The uptake of PrP^{Sc} by HK cells exposed to medium containing iCJD or vCJD brain homogenates monitored by Western blot analysis was also evaluated by densitometry. This was carried out to support the observation that the level of cell associated PrP^{Sc} does increase with the time of cell exposure to prion spiked medium

(Figure 3.27 and 3.28). The assessment method is described in Materials and Methods section (2.10.5).



Densitomerty					
iCJD	No.	Label	Type	Volume (Int)	Background normalised data
1h	1	U1	Unknown	467149815	31090897
3h	2	U2	Unknown	469852866	33793948
6h	3	U3	Unknown	517107866	81048948
24h	4	U4	Unknown	675679597	239620679
48h	5	U5	Unknown	795703942	359645024
	6	B1	Background	436058918	
vCJD	No.	Label	Type	Volume (Int)	Background normalised data
1h	1	U1	Unknown	513863571	62650013
3h	2	U2	Unknown	518680504	67466946
6h	3	U3	Unknown	841916030	390702472
24h	4	U4	Unknown	979094394	527880836
48h	5	U5	Unknown	930329936	479116378
	6	B1	Background	451213558	

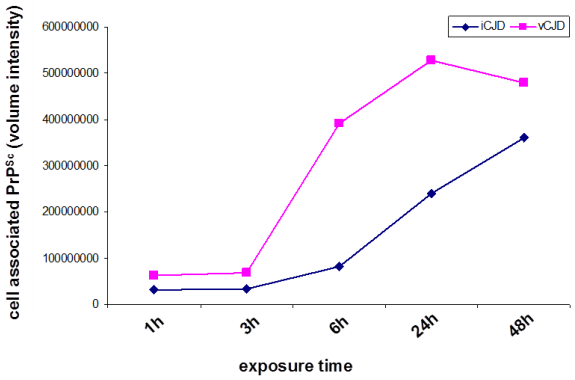
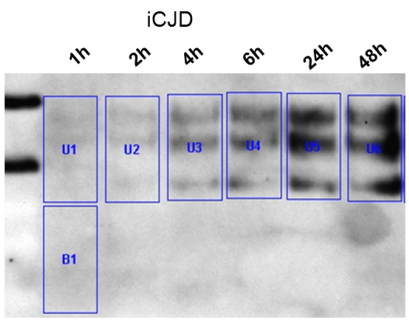


Figure 3.27: Assessment of the time-course of PrP^{Sc} uptake by HK cells after incubation with iCJD or vCJD brain homogenates
(A) Western blot analysis of the the PrP^{Sc} uptake by HK cells in the period of 48 hours exposure to either iCJD or vCJD brain homogenates. (B) PrP^{Sc} signal values assesed by densitometry. (C) The graph represents a quantitative assessment of vaules of PrP^{Sc} signal portrayed as an arbitrary units using Microsoft Office Excel®.

Another example of densitometric assessment of the time-course of PrP^{Sc} uptake by HK cells after incubation with iCJD brain homogenate is shown in Figure 3.28.

A



B

Densitomerty					
	No.	Label	Type	Volume (Int)	Background normalised data
1h	1	U1	Unknown	532288804	9535166
2h	2	U2	Unknown	576853623	54099985
4h	3	U3	Unknown	624283892	101530254
6h	4	U4	Unknown	702262009	179508371
24h	5	U5	Unknown	805507291	282753653
48h	6	U6	Unknown	884634473	361880835
	7	B1	Background	522753638	

C

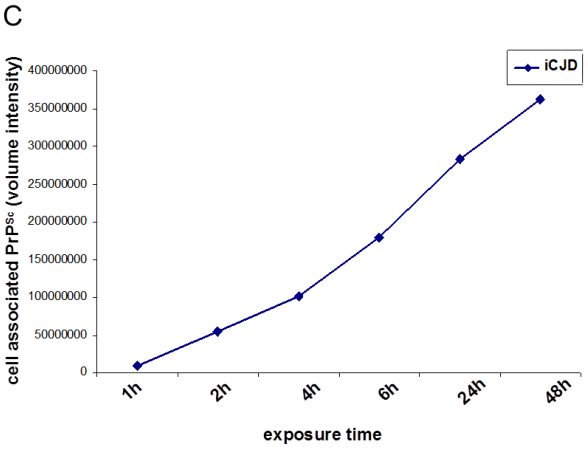


Figure 3.28: Assessment of the time-course of PrP^{Sc} uptake by HK cells after incubation with iCJD brain homogenate

(A) Western blot analysis of the the PrP^{Sc} uptake by HK cells in the period of 48 hours exposure to iCJD brain homogenate. (B) PrP^{Sc} signal values assesed by densitometry. (C) The graph represents a quantitative assessment of vaules of PrP^{Sc} signal portrayed as an arbitrary units using Microsoft Office Excel®.

The densitometric assessments of the Western blots revealed a rising trend of the cell associated PrP^{Sc} signal increasing with the time of HK cells exposure to a medium spiked with prion infected brain homogenates.

3.4.4 Summary

- This study shows that the HK cells exposed to medium containing CJD brain homogenate gradually take up PrP^{Sc}, and presumably prion infectivity, present in the medium.
- The uptake of PrP^{Sc} was rapid and the amount of HK cell-associated PrP^{Sc} increased with time of exposure to the spiked medium. This trend was also confirmed by a densitometric assessment.
- Judged by the gradual uptake and subsequent loss of PrP^{Sc} in the cells exposed to prion infectious medium and then cultured in fresh medium, it appears that the PrP^{Sc} is most likely actively internalised and then either degraded or excreted by the cells.
- Interestingly, the cell-associated PrP^{Sc} signal appeared to be more intense in initial hours of recovery than in the cell lysate just before the removal of infectious medium. Nevertheless, the signal diminished by the 48 hours recovery and was lost completely after the 1^{st} passage.
- Whether an acute new PrP^{Sc} formation can be initiated in these cells without leading to sustainable PrP^{Sc} formation in these cells needs to be investigated further.

3.5 PrP^{Sc} UPTAKE ANALYSED BY IMMUNOCYTOCHEMISTRY

3.5.1 Objectives

To examine the cellular response to acute exposure of prion diseased brain material by immunocytochemistry

3.5.2 Rationale

The study of prion transmission and targeting are major scientific issues with important consequences for public health. Although cells persistently infected with infectious prion agent have provided important insights into the cellular biology of PrP^{Sc} formation during infection, very little is known about how exogenous PrP^{Sc} is initially taken up by the cell.

To address this, the exogenous PrP^{Sc} uptake was earlier analysed by Western blot (3.4). This method provides valuable results, but for a more precise examination and visualisation of how cells (the HK cells and hESC) respond to acute exposure of prion disease brain material, fluorescent immunocytochemistry visualised by confocal microscopy was used. Moreover, the hESC cultures are expensive and time-consuming to prepare in bulk and analysing them by ICC was considered to be a practical and highly informative alternative to Western blot.

The PrP^{Sc} uptake by HK cells and hESC analysed by ICC shown below is representative of more than twelve independent identical and non-identical experiments. The differences included the time points assayed, the origin of the inoculum and the use of pre-treatments during the immunocytochemical procedure (not all data are shown).

3.5.3 Experimental results

3.5.3.1 Immunocytochemistry as a tool for analysing the cell response to exposure with prion infected brain homogenates

For the investigation of the exogenous PrP uptake by hESC or HK cells following experimental design was applied (Figure 3.29). Cells were plated one day before exposure at low density as immunocytochemical analysis is more clear and informative in cells which are spread out rather than tightly packed next to each other. The inocula were composed of sonicated brain homogenate (BSE, vCJD, iCJD, sCJD, AD, bovine negative) diluted to 1% with complete culture medium. The sonication step was applied to break down the PrP^{Sc} aggregate size as this can strongly affect the efficiency of cellular uptake, the mode of endocytosis and the subsequent efficiency of particle processing along the endocytic pathway (Magalhaes *et al.*, 2005; Baron *et al.*, 2006; Greil *et al.*, 2008) (our previous Western blot observations). Cells were exposed to spiked medium for the desired time period and then extensively washed and immunostained for PrP (as described in section 2.12) with one of the anti-PrP protein primary antibodies 3F4 (recognising human amino acid residues 109-112), 6H4 (recognising PrP residues 142-155) or 8H4 (recognising human PrP epitope 175-185). The slides were then examined by fluorescent confocal microscopy. It is important to note that the photomicrographs presented in this thesis truly reflect the overall situation of the whole cell culture population observed in the analysed well.

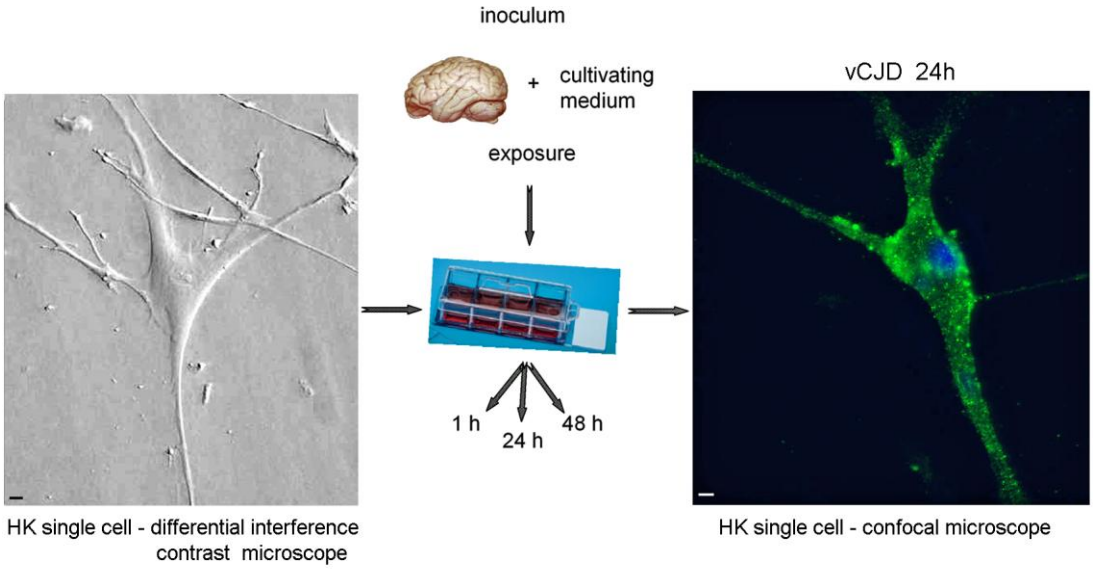


Figure 3.29: Immunocytochemistry as a tool for analysing the cell response to exposure with prion diseased brain homogenates
Cells were plated onto glass chamber slides one day before exposure to brain spiked medium. The 10% prion diseased brain homogenates were sonicated to reduce aggregate size and diluted in complete cell culture medium to 1% of the brain spiked medium. The cells were then continuously exposed to this prepared inoculum for the desired time and extensively rinsed before immunostaining procedure. Cells were stained; either live (2.12.1); or fixed and permeabilised (2.12.2) (as shown on this figure-right); or fixed, permeabilised and denaturated (2.12.3). Primary anti-PrP antibodies employed in throughout this assay were 8H4, 6H4, or 3F4. Secondary antibody used for detection the PrP was FITC Alexa 488. The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.5.3.2 Morphology of hESC of the RCM-1 cell line, RH1 cell line and the human FDC-like HK cells analysed prior and post exposure to brain spiked medium

Firstly, a general cellular response to exposure of brain material was examined (Figure 3.30). The undifferentiated hESC of the RCM-1 cell line (top row), RH1 cell line (centre row) and the FDC-like HK cells (bottom row) were grown in a control medium (left column) or exposed to vCJD brain spiked medium (right column) for 48 hours. Exposure to brain homogenate did not result in gross changes in cell morphology or viability as judged by regular observation of the cultures using differential interference contrast microscopy and parallel comparison with unexposed

control cultures. Although brain homogenate is likely to contain both trophic and toxic molecular stimuli, no gross observable changes were seen even with prolonged exposure under the conditions used.

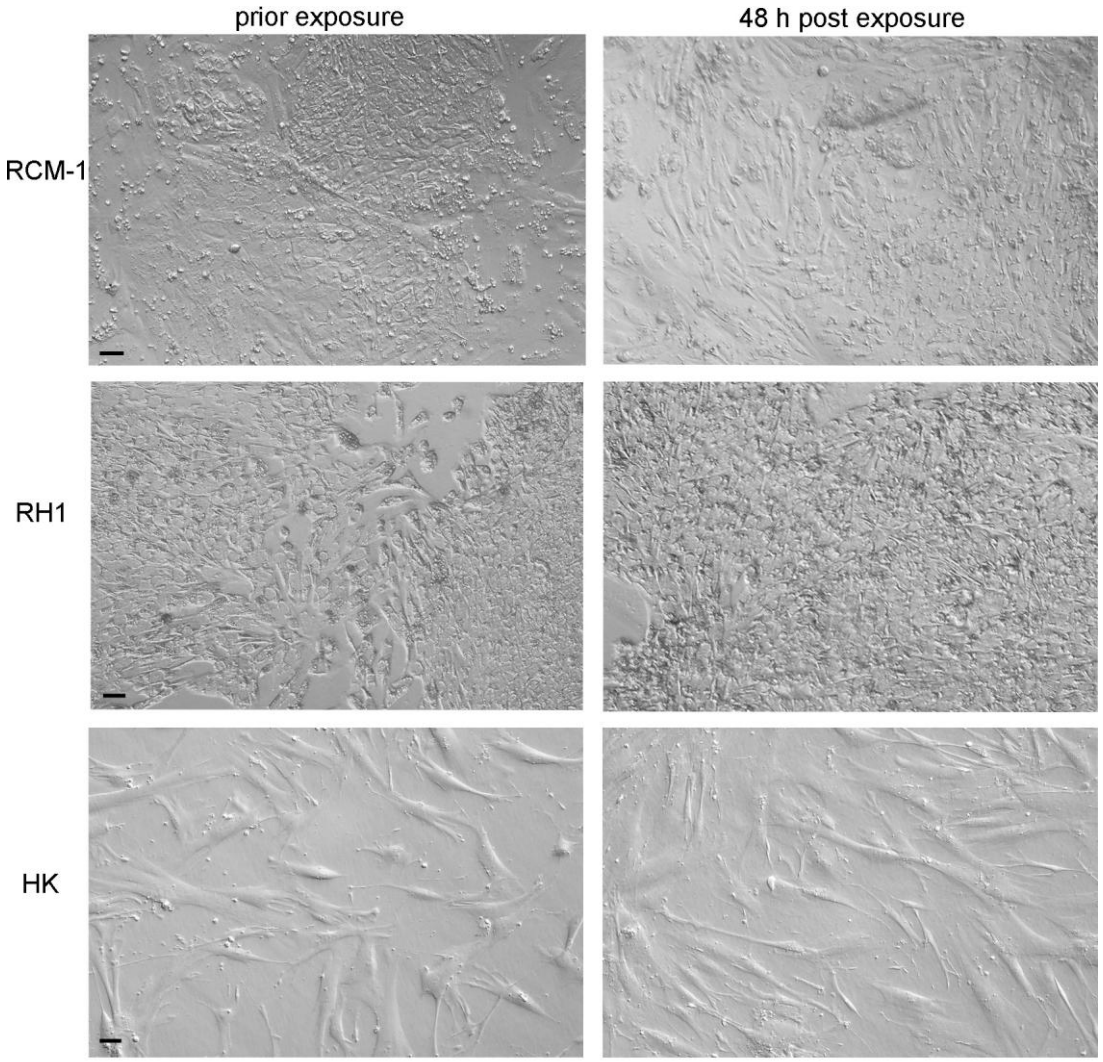


Figure 3.30: Morphology of hESC of the RCM-1 cell line and RH1 cell line and human FDC-like HK cells prior and post exposure to brain spiked medium
The cells were monitored using a differential interference contrast cell culture microscope prior (left column) and post 48 hours incubation with 1% vCJD brain spiked medium (right column). Scale bars (shown in left hand column) represent 50 µm.

3.5.3.3 Adjustment of the signal threshold characteristic for PrP^{Sc}

Due to the lack of PrP^{Sc} specific antibodies to specifically visualise cell-associated exogenous PrP^{Sc}, a denaturation step with guanidine pre-treatment (Gnd) was required. This pre-treatment diminishes PrP^{C} and enhances PrP^{Sc} signal in exposed cells, however it did not enable complete distinction between PrP^{Sc} and PrP^{C}. Therefore, a stringent approach of advanced fluorescence microscopy imaging technology, complemented by quantitative image analysis to discriminate subcellular PrP^{Sc} from PrP^{C} by adjusting the immunofluorescence signal threshold characteristic for PrP^{Sc} in cells exposed to prion infectious brain homogenates was employed (Figure 3.31).

A similar thresholding approach discriminating the lower PrP^{C} fluorescence in order to extract only the higher fluorescence signal of PrP^{Sc} was recently proved useful by Veith and colleagues (Veith *et al.*, 2009).

First, unexposed HK cells were stained without employing a digestion/denaturation step by PK/Gnd pre-treatment (A) or with pre-treatment (B) and the detectable level of PrP^{C} expressed by these cells was visualised. Faint punctuate staining of unexposed HK cells in the absence of the digestion/denaturation pre-treatment was indicative of PrP^{C} (A). Loss of this signal following digestion/denaturation pre-treatment confirmed that the signal is PrP^{C} (B). The HK cells exposed to vCJD spiked medium for 48 hours were also immunostained without (C) or with (D) the PK/Gnd pre-treatment. Extensive diffuse and granular staining of vCJD brain homogenate exposed HK cells in the absence of digestion/denaturation pre-treatment was indicative of mixture of PrP^{Sc} (C-bright green) and PrP^{C} (C-faint green). Very bright granular peri-nuclear staining in vCJD brain homogenate exposed HK cells following digestion/denaturation pre-treatment suggesting specific detection of PrP^{Sc}

under these conditions (D). The analysis of cells exposed to vCJD and pre-treated with PK/Gnd showed the signal intensities characteristic of PrP^{Sc} staining (D) and this threshold was taken in account for further detection of PrP^{Sc} only in cells exposed to prion infected brain material.

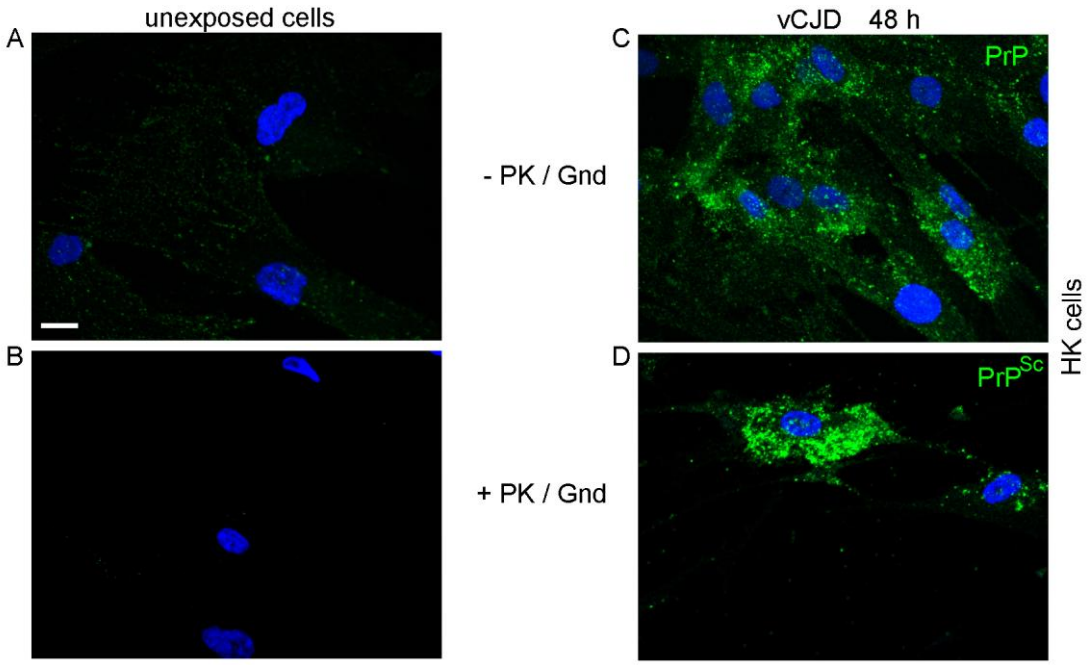


Figure 3.31: Signal threshold characteristic for PrP^{Sc}
Acquisition adjustment to immunostaining procedure allowed us to clearly distinguish between PrP^{C} and PrP^{Sc} signals by immunofluorescence. Control HK cells were cultured in control medium and immunostained without (A) or with denaturation step (PK/Gnd) (B). Cells exposed to 1% vCJD for 48 hours and immunostained without denaturation step (C) and with denaturation step (D). Cells were immunostained for PrP with the antibody 6H4 (green). The nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.5.3.4 Progressive PrP^{Sc} uptake by the hESC of the RCM-1 line continuously exposed to BSE and vCJD brain homogenates analysed by immunocytochemistry

In order to ascertain how hESC respond to acute exposure of infectious brain material and to monitor the fate of exogenous PrP^{Sc} in the culture (Figure 3.32), the hESC of the RCM-1 cell line (*PRNP* codon 129-MM genotype) were continuously exposed to crude sonicated 1% extracts of BSE (A, E, I), vCJD (B, F, J) and AD (C, G, K) brain homogenates, or grown in a control medium (D, H, L) for 1 (A-D), 24 (E-H), and 48 hours (I-L). The presence and the intracellular distribution of PrP^{Sc} within individual cells was then visualised by routine immunostaining employing 8H4 antibody and confocal analysis. Unexposed hESC and those exposed to AD brain homogenate showed no detectable PrP^{C} under the immunostaining conditions used in this experiment. However, RCM-1 cells exposed to BSE (A, E, I) and vCJD (B, F, J) brain material showed intense staining of PrP^{Sc}, increasing with exposure time, suggesting uptake of PrP^{Sc} from the spiked medium.

The staining pattern of cells exposed to BSE or vCJD brain were similar indicating no pronounced species or strain-related selectivity of the internalisation process. PrP^{C} expressed by the hESC was not detectable under these experimental conditions (D, H, L).

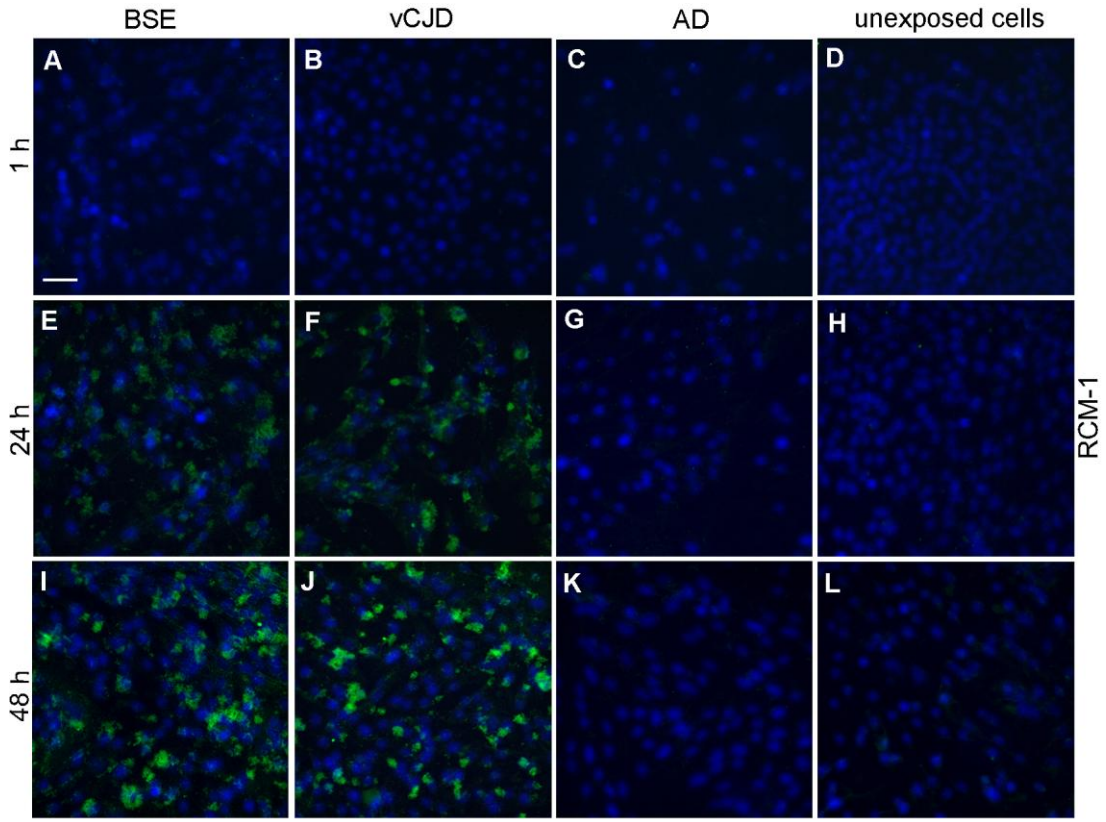


Figure 3.32: Progressive PrP^{Sc} uptake in hESC RCM-1 line continuously exposed to BSE and vCJD brain homogenates analysed by immunocytochemistry
RCM-1 cells (*PRNP* codon 129-MM genotype) were exposed to BSE (A, E, I), vCJD (B, F, J), or AD (C, G, K) 1% brain homogenate, or grown in control medium without brain homogenate (D, H, L) for 1 hour (A-D), 24 hours (E-H) or 48 hours (I-L). The cells were then extensively washed, fixed, permeabilised and immunostained for PrP with the antibody 8H4 recognising human PrP epitope 175-185 (green), and the nuclei counterstained with DAPI (blue) as previously described in section 2.12.2. The scale bar represents 50 µm.

3.5.3.5 Progressive PrP^{Sc} uptake by the hESC of the RH1 line continuously exposed to BSE and vCJD brain homogenate analysed by immunocytochemistry

The results were similar when hESC of the RH1 line (*PRNP* codon 129-MV genotype) (Figure 3.33) were exposed to BSE (A, E, I), vCJD (B, F, J), AD (C, G, K) or grown in control medium (D, H, L). The pattern of PrP^{Sc} immunostaining was observed in both vCJD and BSE, increasing with exposure time, as previously observed in RCM-1 line.

PrP^{Sc} uptake into the undifferentiated human embryonic stem cells for each given time point showed that in both cell lines, there were no apparent differences in PrP^{Sc} uptake amongst the different prion strains. It seemed that majority of the cells in cultures after exposure to either BSE or vCJD were positive for cell associated PrP^{Sc}, indicating that a major subpopulation of the cells is able to take up the exogenous PrP^{Sc} material.

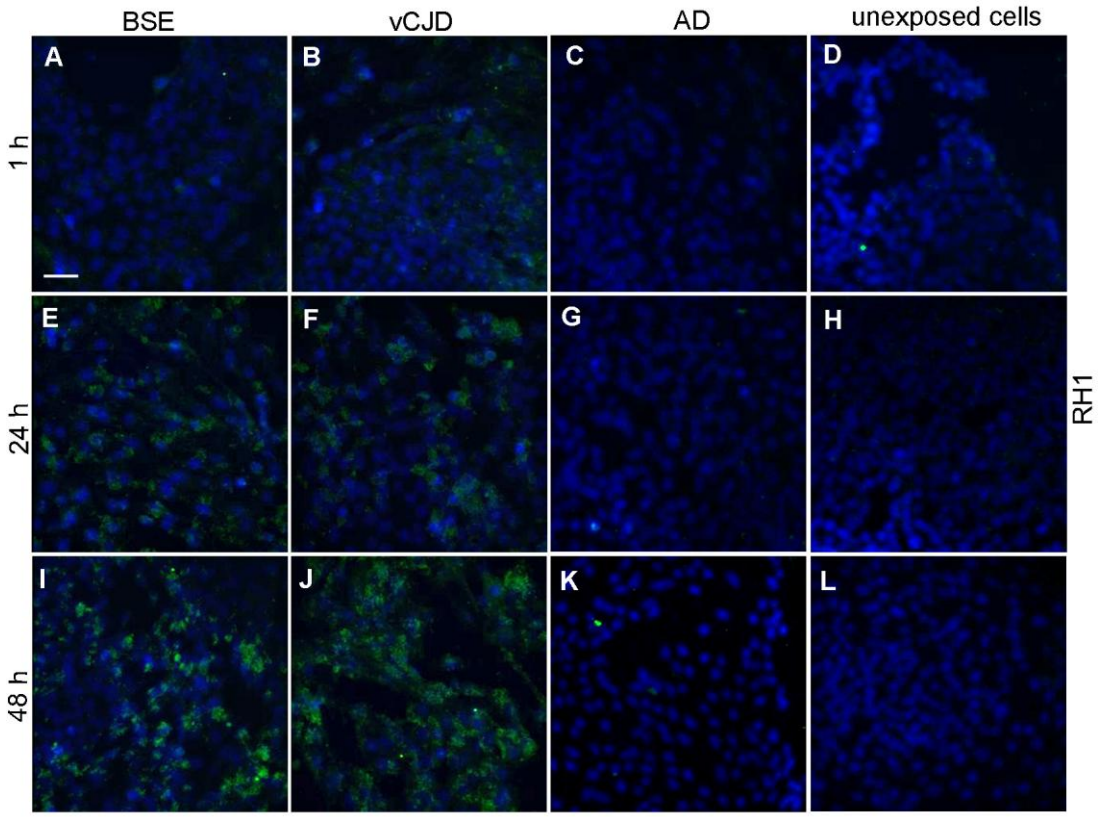


Figure 3.33: Progressive PrP^{Sc} uptake in hESC RH1 line continuously exposed to BSE and vCJD brain homogenate analysed by immunocytochemistry
RH1 cells (*PRNP* codon 129-MV genotype) were exposed to BSE (A, E, I), vCJD (B, F, J), or AD (C, G, K) 1% brain homogenate, or grown in control medium without brain homogenate (D, H, L) for 1 hour (A-D), 24 hours (E-H) or 48 hours (I-L). The cells were then extensively washed, fixed, permeabilised and immunostained for PrP with the antibody 8H4 (green), and the nuclei counterstained with DAPI (blue). The scale bar represents 50 µm.

3.5.3.6 Undifferentiated hESC retain their pluripotent state after being exposed to brain homogenate

To ascertain how the undifferentiated human embryonic stem cells react to exposure of infectious brain material, their pluripotent state was examined at 48 hours post exposure to prion infectious brain material (Figure 3.34). To put it briefly, the hESC of the RCM-1 line were exposed to vCJD spiked medium for 48 hours. Then the medium was removed, the cells were washed, fixed, permeabilised and double immunolabeled for PrP using the 6H4 antibody (A) and the NANOG antibody detecting stem cell pluripotency factor (B).

The stem cell pluripotency factor is only expressed in nucleus of stem cells retaining their undifferentiated phenotype (Pan and Thomson, 2007). The merge of the channels is shown in (C). The majority of cells which showed positive intracellular PrP^{Sc} immunostaining (suggesting uptake of exogenous PrP^{Sc}) (green, A-arrow) also displayed the pluripotency factor in their nucleus (pink, B-arrow). Stem cell cultures are, however, not homogeneous and do produce partially differentiated derivative cells as a normal part of their growth. These cells do not produce pluripotency factor any more and are negative for staining with NANOG (blue, B). The merge (red, C) indicates that majority of cells positive for PrP^{Sc} uptake were the undifferentiated hESC (arrow), and exposure to brain material did not encourage them to differentiate towards a random phenotype in the observed 48 hours of exposure.

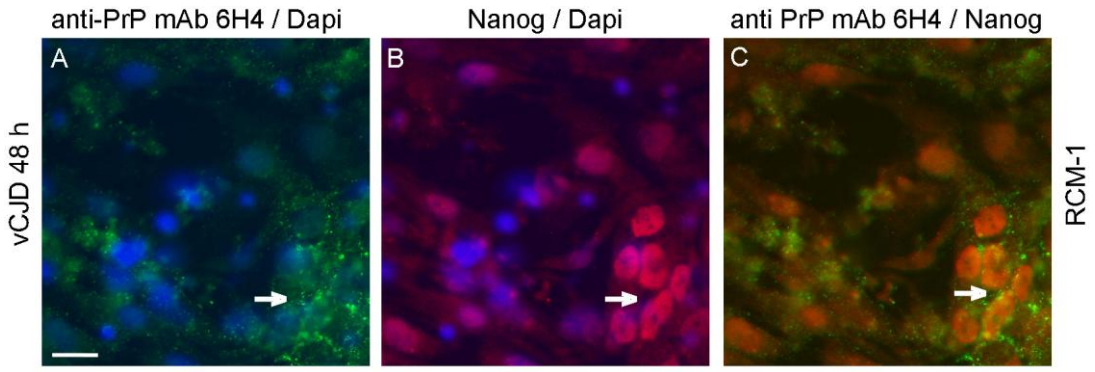


Figure 3.34: Undifferentiated hESC retain their pluripotent state after being exposed to brain homogenate
The RCM-1 cell line was exposed to 1% vCJD brain spiked medium for 48 hours. The cells were then washed, fixed, permeabilised and immunostained for PrP with mAb 6H4 (A) (green) and stem cell pluripotency marker NANOG (B) (pink) as described in section 2.12.4. Merge of FITC (PrP^{Sc}) and red (NANOG) channels is shown in (C). The cells' nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.5.3.7 PrP^{Sc} uptake in extraneuronal lymhoreticular FDC-like HK cell line continuously exposed to iCJD, sCJD and vCJD brain homogenates analysed by immunocytochemistry

To visualise how the HK cells (*PRNP* codon 129-VV genotype) respond to acute exposure of infectious brain material, the fate of PrP^{Sc} added to these culture was also monitored by immunocytochemistry (Figure 3.35). Cells were exposed to crude sonicated extracts of $iCJD_1$ (A), $sCJD_3$ (B), vCJD (C), AD (D) or cultured unchallenged (E) for 48 hours. Then the cells were fixed, permeabilised and immunostained by routine immunocytochemistry. At the 48 hours time point, an apparently similar PrP^{Sc} immunostaining signal could be observed in cells exposed to iCJD, sCJD, or vCJD (A, B, C, respectively). Unexposed HK cells (E) and those exposed to AD brain homogenate (D) showed faintly detectable cell own PrP^{C} under the immunostaining/confocal microscopy conditions used in this experiment.

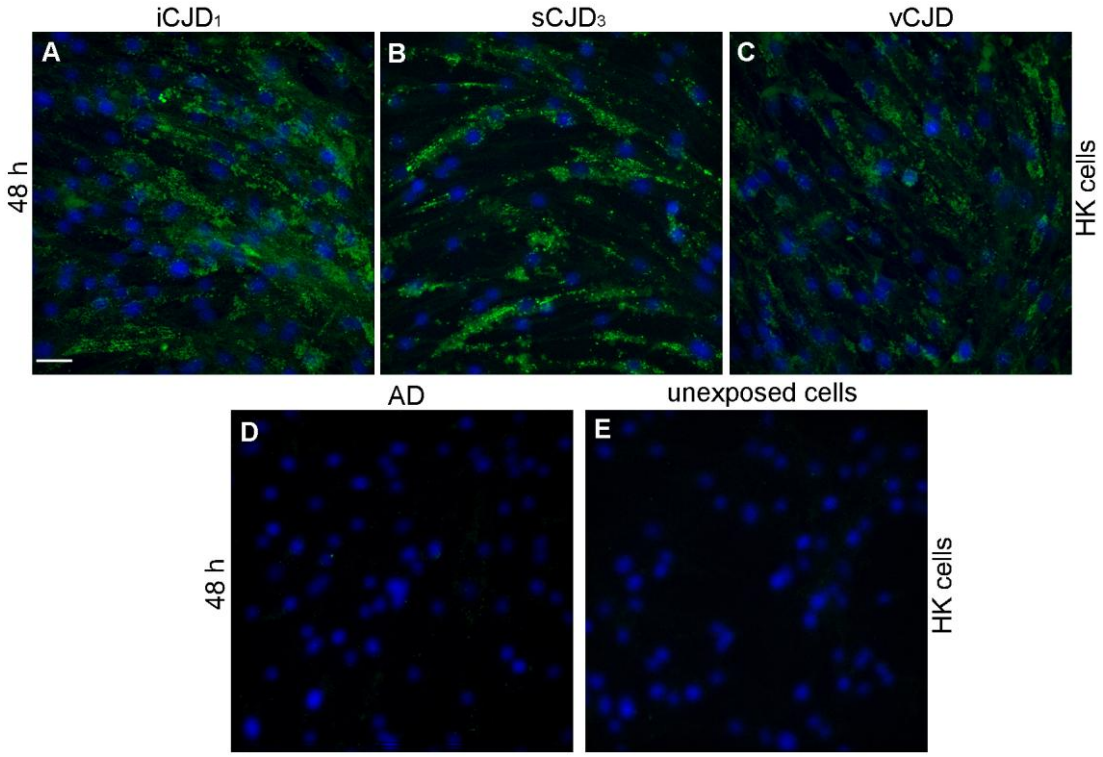


Figure 3.35: PrP^{Sc} uptake in extraneuronal lymhoreticular FDC-like HK cell line continuously exposed to iCJD, sCJD and vCJD brain homogenates analysed by immunocytochemistry

HK cells (*PRNP* codon 129-VV genotype) were exposed to $iCJD_1$ (A), $sCJD_3$ (B), vCJD (C), AD (D) 1% brain homogenate, or grown in control medium without brain homogenate (E) for 48 hours. The cells were then fixed, permeabilised and immunostained for PrP with the antibody 8H4 (green), and the nuclei were counterstained with DAPI (blue) as previously described in section 2.12.2. The scale bar represents 50 µm.

3.5.3.8 Analysis of the HK cell line exposed to BSE-positive and BSE-negative brain homogenates

In next assay was investigated the HK cells response to BSE and bovine (BSE-negative) brain homogenate (Figure 3.36) after 24 hours of continuous exposure. The bovine brain homogenate was used as an additional negative control for exogenous PrP^{Sc} uptake experiments alongside the non-CJD (Alzheimer diseased, AD) brain homogenate used earlier. The cells were fixed, permeabilised and immunolabeled with 8H4 antibody using a routine immunocytochemistry procedure. HK cells challenged with BSE showed intense immunostaining of PrP^{Sc} (Figure 3.36; A) whereas cells challenged with bovine brain material showed no detectable PrP^{Sc} (Figure 3.36; B).

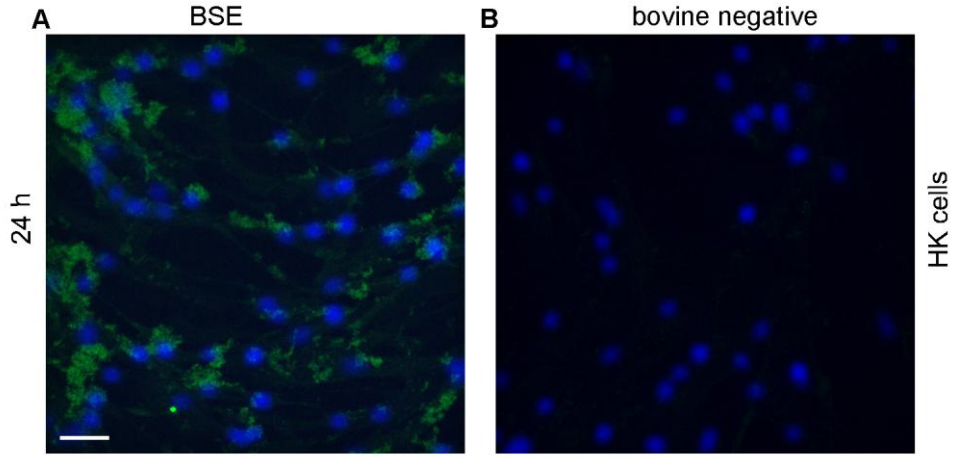


Figure 3.36: HK cell line exposed to BSE and bovine negative brain homogenates
HK cells analysed by immunocytochemistry after 24 hours of continuous exposure to 1% BSE brain spiked medium (A) and 1% bovine (BSE-negative) medium (B). The cells were then extensively washed, fixed, permeabilised and immunostained for PrP with the mAb 8H4 (green). The nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.

3.5.3.9 Morphology of PrP^{Sc} immunostaining observed at a high magnification in RCM-1 and HK cells continuously exposed to vCJD brain spiked medium for 24 hours

To gain insight into the precise localisation of exogenous PrP^{Sc} in cells exposed to infectious brain material, the immunostained cells were examined at high magnification (Figure 3.37 and 3.38). Both cell types (Figure 3.37), the undifferentiated hESC (A, B) and the FDC-like HK cells (C, D), were investigated. After 1 day in culture, cells were incubated with vCJD brain homogenate (A, C) or grown in control medium (B, D) for 24 hours and immunostained with either the 8H4 (A, B) or 6H4 (C, D) using standard protocols of fixation and permeabilisation. Examination of the challenged cells by high magnification fluorescent microscopy revealed the majority of the granular staining of PrP^{Sc} was found at perinuclear regions (A, C). The PrP^{Sc} immunostaining of exposed cells was very intense, when compared to the fainter patchy presumably PrP^{C} immunostaining of the unexposed cells (B, D).

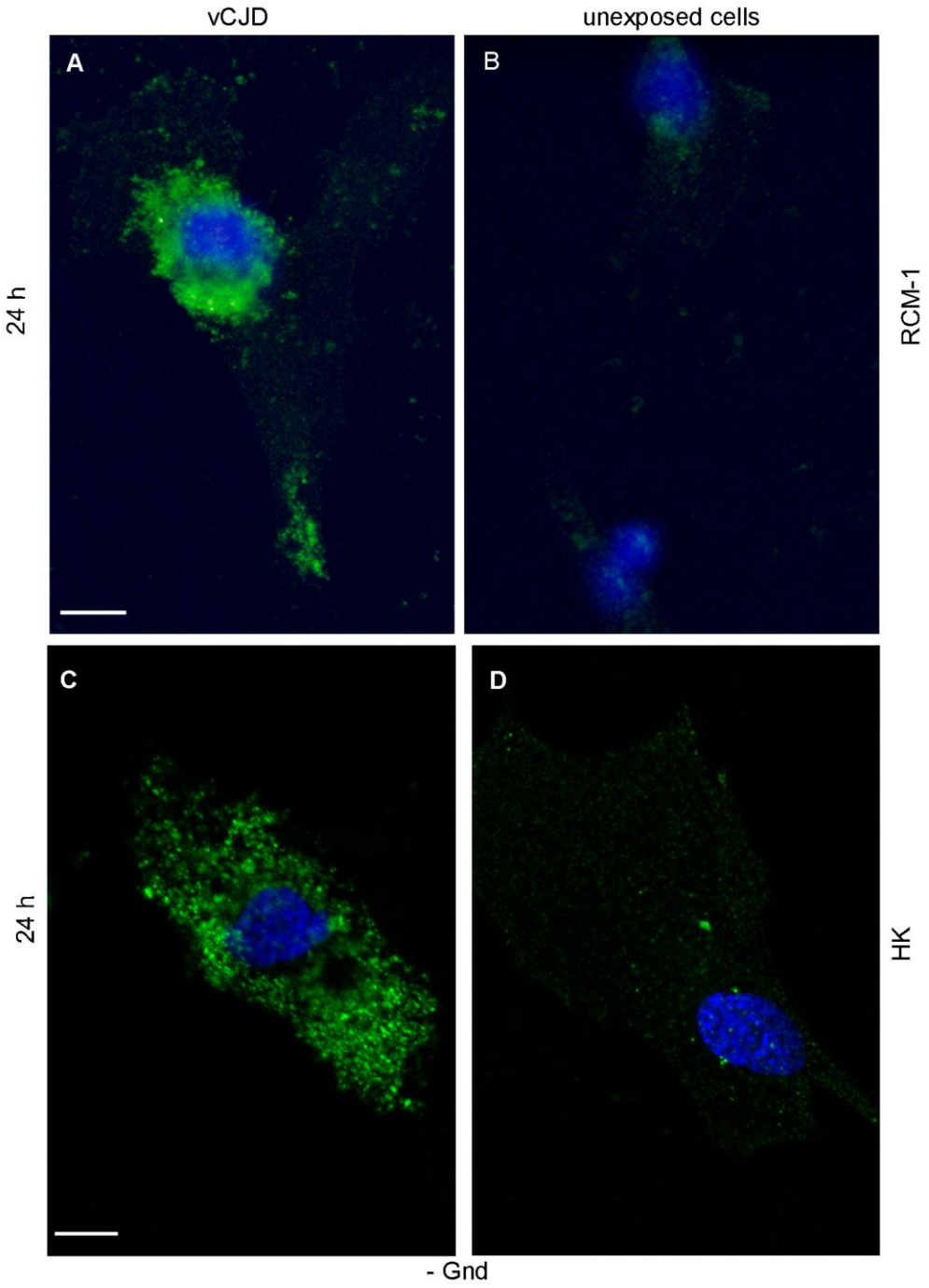


Figure 3.37: Morphology of PrP^{Sc} immunostaining observed at a high magnification in RCM-1 and HK cells continuously exposed to vCJD brain spiked medium for 24 hours
The hESC RCM-1 cells (A, B) and FDC-like HK cells (C, D) were exposed to 1% vCJD brain spiked medium (A, C) or grown in control medium (B, D) for 24 hours. The cells were then washed, fixed, permeabilised and immunostained for PrP with the antibody 8H4 (A, B) and 6H4 (C, D) (green). The nuclei were counterstained with DAPI (blue). The scale bars represent 20 µm.

3.5.3.10 Morphology of PrP^{Sc} immunostaining observed at a high magnification in HK cells continuously exposed to vCJD brain spiked medium for 48 or 72 hours

Next, the PrP^{Sc} distribution profile in HK cells continuously exposed to vCJD spiked medium for 48 (Figure 3.38; A) and 72 (Figure 3.38; B) hours was analysed. As identical results were obtained with either the 8H4 or 6H4 anti-PrP antibody in previous assays, the 6H4 antibody was further used in most of the experiments. The immunostained cells were analysed by high magnification fluorescent microscopy. The PrP^{Sc} at 48 and 72 hours was found to be intracellular, as previously observed, with strong perinuclear accumulation with a coarse granular morphology. A faint patchy PrP^{C} signal could be observed at the periphery of the HK cell cytoplasm. It is important to note that a prominent juxtanuclear deposition pattern of the PrP^{Sc} taken up by cells exposed to various prion disease brain homogenates (BSE, vCJD, iCJD, sCJD) was typically observed.

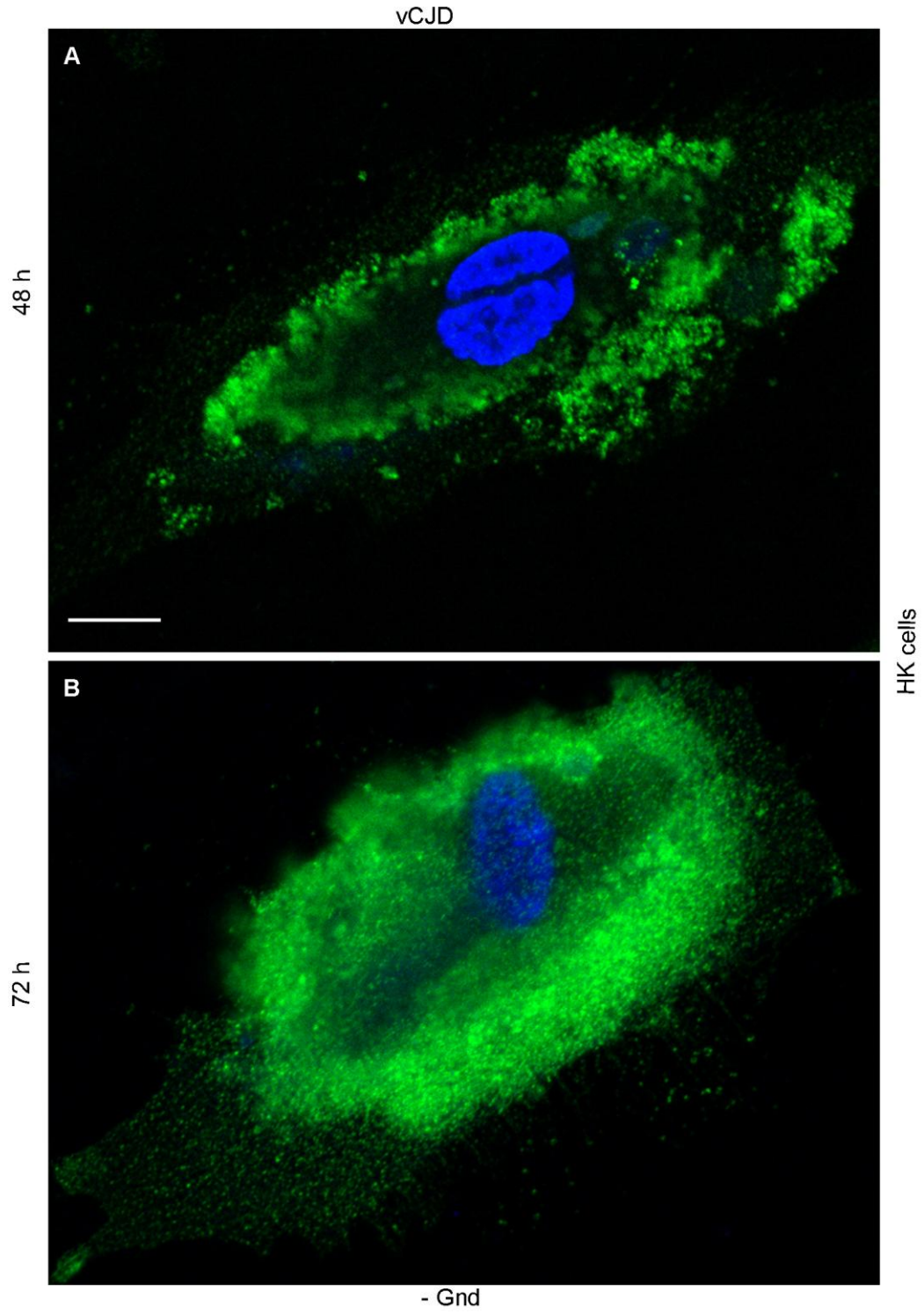


Figure 3.38: Morphology of PrP^{Sc} immunostaining observed at a high magnification in HK cells continuously exposed to vCJD brain spiked medium for 48 or 72 hours
HK cells were continuously incubated with 1% vCJD brain spiked medium for 48 hours (A) and 72 hours (B). The cells were then washed, fixed, permeabilised and immunostained for PrP with the antibody 6H4 (green). The nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.5.3.11 Immunofluorescent staining of non-permeabilised and non-denatured living hESC and FDC-like cells after incubation with brain spiked medium

In order to eliminate the possibility that the immunostaining patterns obtained depend upon brain material, including PrP^{Sc}, adhering to the cell surface, but is rather internalised via an active cellular process such as endocytosis, staining of living cells was conducted with hESC of the RCM-1 line and the FDC-like HK cells exposed to infectious brain material (Figure 3.39). Immunocytochemistry of fixed and permeabilised hESC and HK cells exposed to prion infectious brain material previously resulted in a strong PrP^{Sc} immunostaining signal. However, the non-permeabilised and non-denatured live cells only showed a very faint immunostaining signal (Figure 3.39; B) which is thought to be the cell's own PrP^{C} anchored at the plasma membrane.

The hESC of the RCM-1 line (A) and FDC-like HK cells (B) were exposed to BSE (Aa), $iCJD_1$ (Ba), vCJD (b), AD (c) brain spiked medium or grown in a control medium (d) for 48 hours. Then the brain spiked medium was removed, the cells were washed, blocked and immunolabeled with anti-PrP antibody 8H4 (A), or 6H4 (B).

Confocal fluorescence microscopy of live cells showed no immunostaining of undifferentiated hESC (A), or only a very faint patchy signal of consistent with PrP^{C} in HK cells when viewed at higher magnification (B). Therefore, live cell immunostaining confirmed that the earlier observed PrP^{Sc} immunostaining signal of cells exposed to infectious brain material was indeed cytoplasmic rather than material attached or deposited on the cell surface.

It is important to note that immunostaining of live cells resulted in an analysis of fewer cells when compared to immunostaining of fixed cells. This was a simple matter of unfixed cells not being strongly adhered to the slide and being washed off

during the immunostaining procedure. Also, these cells had to be analysed on the day of immunolabeling as they easily sensitised to apoptotic insults and were therefore not analysable.

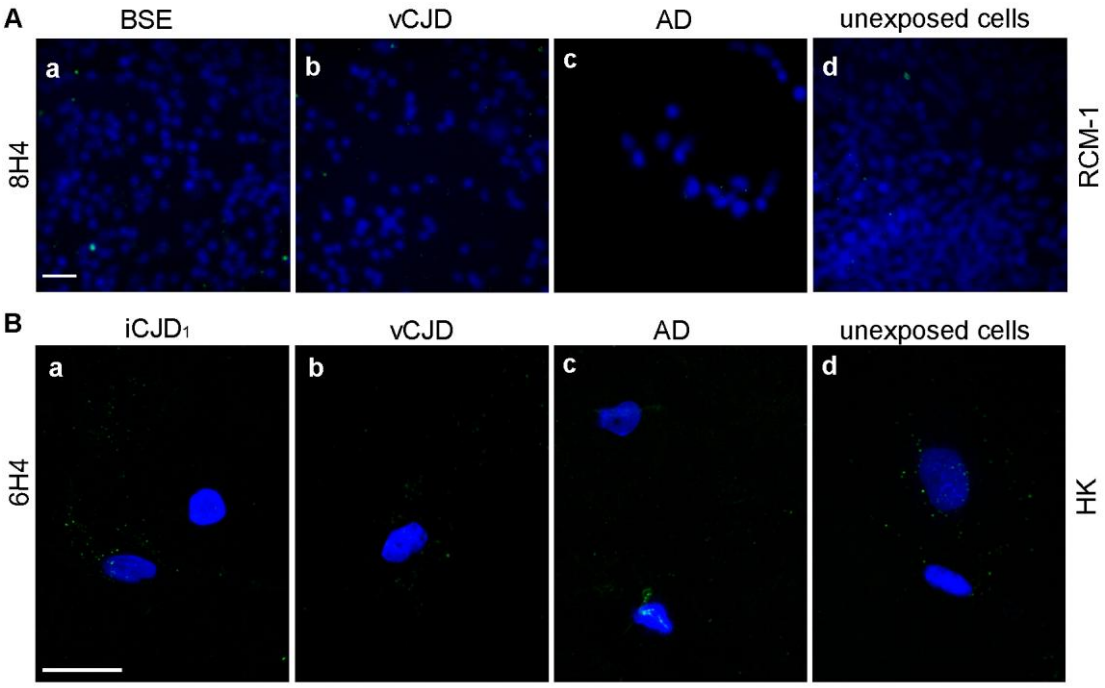


Figure 3.39: Immunofluorescent staining of non-permeabilised and non-denatured living hESC and FDC-like cells after incubation with brain spiked medium
(A) hESC of the RCM-1 line were exposed to 1% BSE (a), vCJD (b), AD (c) or grown in control medium (d) for 48 hours. The cells were then washed and immunostained as described in section 2.12.1 with anti-PrP monoclonal antibody 8H4. (B) Immunostaining of living HK cells with anti-PrP monoclonal antibody 6H4 showed very faint signal on the surface of cells challenged with infectious brain material (a, b), AD (c) brain material and those left unchallenged (d). The cells' nuclei were counterstained with DAPI (blue) and the scale bars represent 50 µm.

3.5.3.12 Optical sectioning of HK cell exposed to vCJD brain material

To visualise and confirm the intracellular localisation of exogenous PrP^{Sc} taken up by cells exposed to prion diseased brain homogenates, confocal microscopy Z-stack sections were performed (Figure 3.40). This method (optical sectioning) samples a series of planes within the cell separately, which can then be assembled into a series or Z-stack. HK cells were exposed to vCJD brain homogenate for 24 hours and immunolabeled with 8H4 anti-PrP antibody after standard conditions of fixation and permeabilisation. The three selected Z-stack sections, the bottom of the cell (left), middle of the cell (centre) and top of the cell (right), confirmed that the PrP immunostained material in prion disease brain homogenate exposed cells is indeed present within the cell and accumulated in the juxtanuclear area of the cell.

Note in the bottom Z-stack section (left) the periphery of the HK cell cytoplasm is indicated by a faint punctuated PrP^{C} signal.

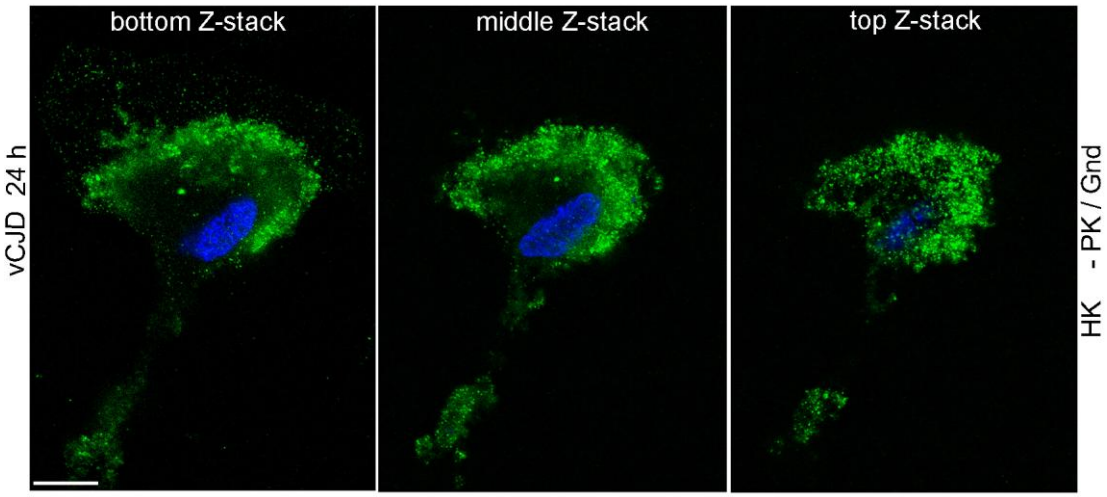


Figure 3.40: Optical sectioning of HK cell exposed to vCJD brain material
The exogenous PrP^{Sc} taken up by HK cells exposed to 1% vCJD for 24 hours was visualised by Z-stack (optical sectioning) allowing insight into various depths throughout the immunostained cell – bottom (left), middle (centre) and top (right) sections of the exposed cell are shown. The cells were washed, fixed, permeabilised and immunostained for PrP with the antibody 8H4 (green). The cells' nuclei were counterstained with DAPI (blue) and the scale bar (left) is 20 µm.

3.5.3.13 Western blot analysis of spin filtered and crude brain homogenates

The next experiment examined whether different particle sizes of exogenous PrP^{Sc} would alter the uptake and intracellular site of PrP^{Sc} accumulation (Figure 3.41). The BSE brain material was prepared as a 10% w/v homogenate using a ribolyser (as described in section 2.11.1.2). The homogenate was then cleared and the supernatant was further sonicated and then either filtered through 220 nm (Figure 3.41; lane 1), 450 nm (Figure 3.41; lane 2) spin filters or allowed to remain as a cleared unfiltered homogenate (Figure 3.41; lane 3). As expected, filtration resulted in a decrease of PrP^{Sc} in the brain homogenate (lane 1 and 2). The 220 nm pore sized spin filter removed a large proportion of the PrP^{Sc} present in the cleared homogenate, although a small amount of PrP^{Sc} was still detectable in the filtrate.

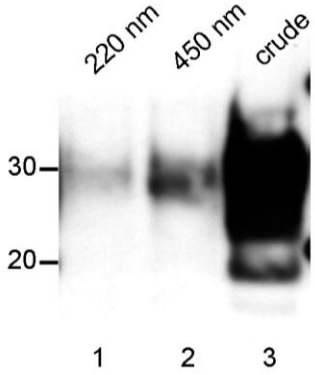


Figure 3.41: Western blot analysis of spin filtered and crude brain homogenates
The 10% brain homogenates (Western blot of BSE is shown) were rybolised and sonicated (as described in section 2.11.1.2) in order to break down the PrP^{Sc} aggregates, then spin filtered using 220 nm (lane 1) or 450 nm (lane 2) spin filters by centrifugation at 10,000 rpm for 5 minutes or retained crude (lane 3). Then the samples were treated with PK (50 µg/ml) and analysed by standard Western blot with anti-PrP primary antibody 6H4. The quantity of individual brain homogenates loaded onto gel was equivalent. The molecular weight, in kDa, is marked left.

3.5.3.14 HK cells exposed to filtered and crude vCJD brain spiked medium

Next, the above prepared homogenates were diluted to concentration of 1% with complete cell culture media and continuously incubated with the HK cells for 48 hours. Then the medium was withdrawn, and the cells were extensively washed, fixed, permeabilised, immunolabeled with the anti-PrP antibody 6H4 and examined with confocal microscopy (Figure 3.42). The intensity of the PrP immunostaining signal (bright green) corresponded to the quantity of PrP^{Sc} remained in the vCJD homogenate after filtration with either 220 nm (B) or 450 nm (C) pore sized spin filters and in the crude brain homogenate (D). The PrP^{Sc} immunostaining resulted in the typical accumulation site at the juxtanuclear area in cells exposed to either filtered, or crude spiking material (B-D). Interestingly, the immunostaining signal of PrP^{Sc} in cells exposed to vCJD brain spiked medium filtered through the 220 nm spin filter (B) was finer when compared to the typical coarse granular morphology of PrP^{Sc} immunostaining in cells exposed to the crude vCJD brain homogenate (D). The unexposed control cells are shown in (A). Note the periphery of the HK cell is visualised by faint patchy PrP^{C} signal.

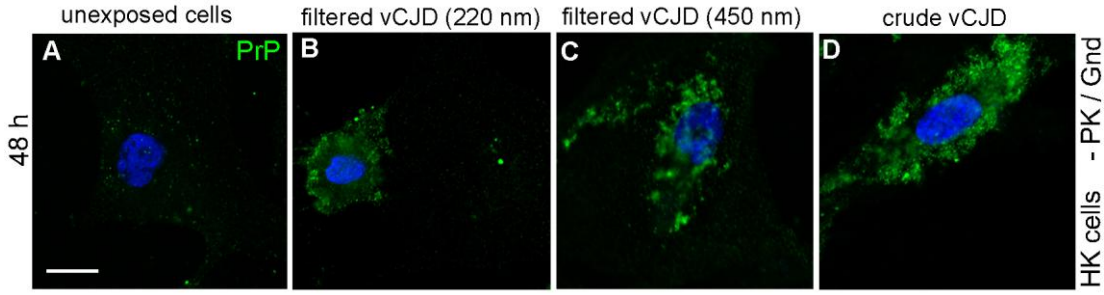


Figure 3.42: PrP^{Sc} uptake from filtered vCJD brain spiked medium by the HK cells
PrP^{Sc} from vCJD filtered through spin filters of pore size 220 nm (B) or 450 nm (C) or crude brain homogenate (D) adopt the same perinuclear localisation after being taken up by HK cells. The cells were continuously exposed to the brain homogenates for 48 hours, then fixed, permeabilised and immunolabeled for PrP with 6H4 primary antibody. Control unexposed cells (A) showing cell's faint patchy PrP^{C} immunostaining. DAPI staining (blue) locates nuclei. Scale bar (left), 20 µm.

3.5.3.15 HK cell survival and morphology monitoring after immunostaining procedure using proteinase K and guanidine thiocyanate pre-treatments

Up to this point it was possible to distinguish PrP^{Sc} from PrP^{C} by monitoring the characteristic fluorescent signal threshold in the quantitative confocal image analysis system. But to specifically recognise only PrP^{Sc}, a digestion/denaturation step with proteinase K (PK) and guanidine thiocyanate (Gnd) was employed (Taraboulos *et al.*, 1990). Optimisation of the standard immunocytochemistry protocol was performed by testing variations of the denaturant concentrations and times of pre-treatments resulting in specifically PrP^{Sc} detection and exposing PrP^{Sc} epitopes that were previously masked (Figure 3.43). Cell morphology was carefully monitored after each pre-treatment step and recorded. The final optimised protocol was composed of extensive washes with PBS (A), fixation with 4% paraformaldehyde (B), permeabilisation with 0.1% Triton X-100 (C), denaturation with very low concentration of proteinase K (0.3 µg/ml) (D), subsequent pre-treatment with 4 M guanidine thiocyanate (E), blocking with 3% BSA (F), incubation with anti-PrP primary antibody (G) followed by incubation with FITC secondary antibody (H).

Although PK digestion is a harsh procedure, the majority of the cells remained intact. The result using a protocol in which only Gnd pre-treatment (without PK treatment) was used, is shown in (I). This methodological variation became useful in experiments where cell organelle structural integrity needed to be preserved (3.7-8).

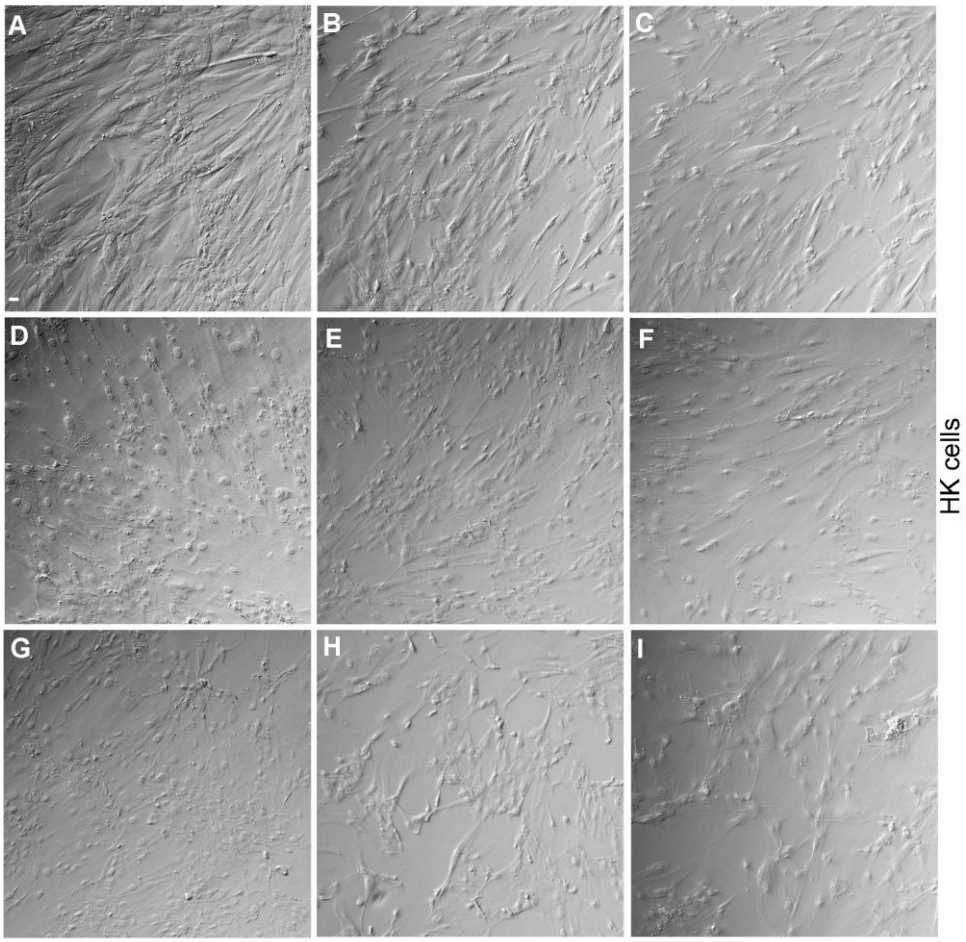


Figure 3.43: HK cell survival and morphology monitoring after immunostaining procedure using proteinase K and guanidine thiocyanate pre-treatments
The cellular response was carefully monitored with the differential interference contrast culture microscope after each step of the immunostaining procedure. Cell morphology after (A) extensive wash with PBS, (B) incubation with 4% paraformaldehyde (PFA), (C) treatment with 0.1% Triton X-100, (D) treatment with proteinase K (PK) (0.3 µg/ml), (E) treatment with 4 M guanidine thiocyanate (Gnd), (F) blocking with 3% bovine serum albumin (BSA), (G) incubation with primary mAb, (H) incubation with secondary Ab. Cell morphology where treatment with PK was excluded from protocol and only treatment with Gnd was carried out is shown in (I). The scale bar shown in (A) corresponds to 50 µm.

3.5.3.16 Confirmation that PrP^{Sc} signal corresponded to exogenous PrP^{Sc} taken up by exposed cells

In order to confirm whether the PrP immunostaining results upon the cellular uptake of exogenous PrP^{Sc} from the brain spiked media, or whether the signal is a result of already established infection, we took advantage of immunocytochemical pre-treatment with PK and Gnd and use of two anti-PrP primary antibodies 6H4 and 3F4 (Figure 3.44). The benefit in using the 3F4 antibody is that it recognises human, but not bovine PrP.

Cells of either hESC RCM-1 line (A) or the FDC-like HK cell line (B) were exposed to BSE (a, d), vCJD (b, e) or AD (c, f) brain spiked medium for 48 hours. The cells were subsequently fixed, permeabilised, PK and Gnd pre-treated and immunostained with anti-PrP primary antibodies 6H4 (a-c) or 3F4 (d-f).

Positive PrP^{Sc} immunostaining signal was observed in cells exposed to vCJD brain spiked medium in both cell types (b, e). The absence of immunostaining in BSE exposed cells with 3F4 (d) confirmed that the immunostaining with antibody 6H4 (a, b) resulted upon uptake of exogenous PrP^{Sc} from the spiked media by challenged cells, rather than from endogenous human cellular PrP^{C} already converted into PrP^{Sc} as a result of BSE exposure. Cells exposed to AD brain material (c, f) remained negative as expected (a few background spots were present).

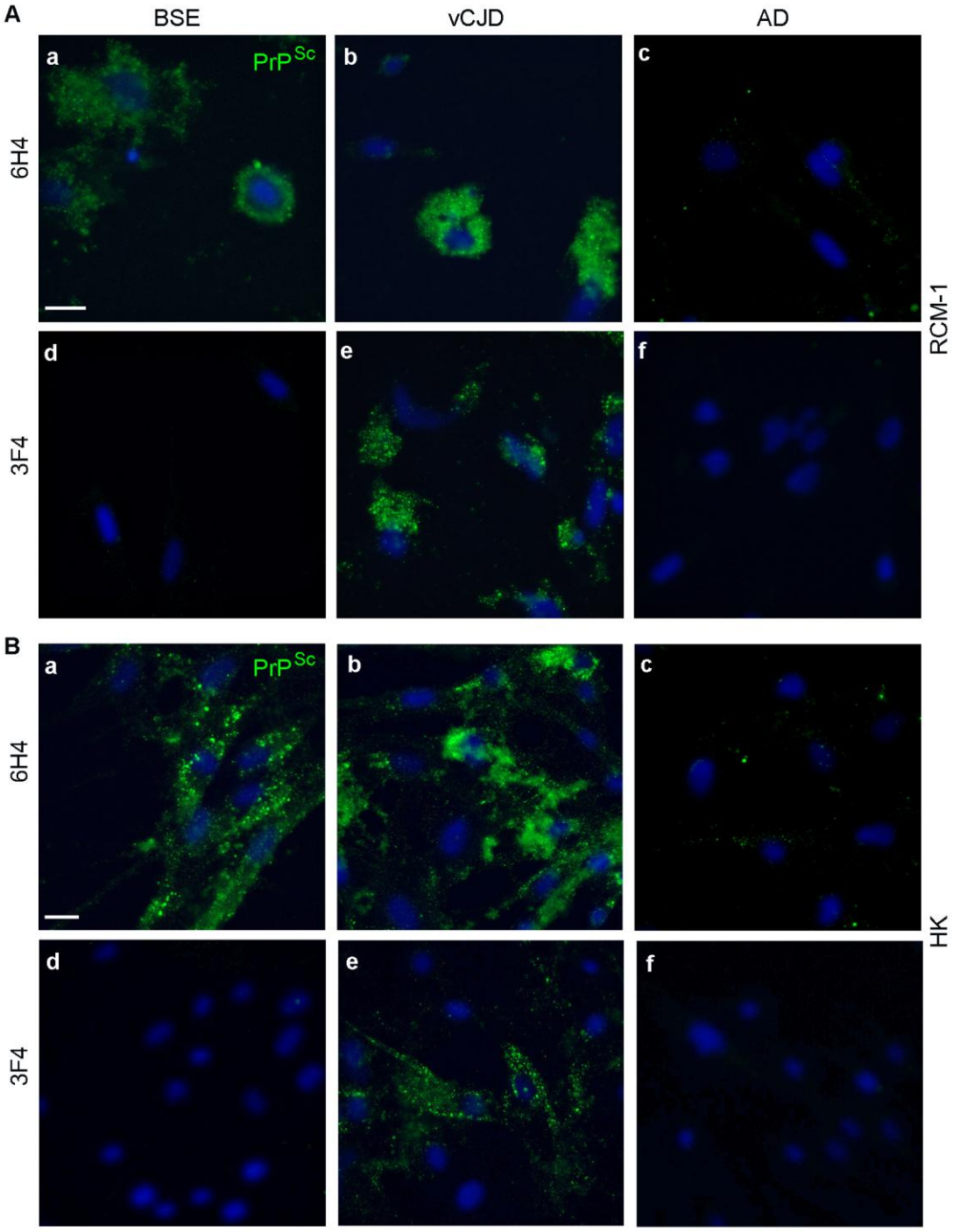


Figure 3.44: Confirmation that PrP^{Sc} signal corresponded to exogenous PrP^{Sc} taken up by exposed cells

(A) hESC of the RCM-1 cell line (*PRNP* codon 129-MM genotype) and (B) the HK cells (*PRNP* codon 129-VV genotype) were exposed to BSE (a, d), vCJD (b, e) or AD (c, f) 1% brain homogenate for 48 hours. The cells were then fixed, permeabilised, treated with PK and Gnd and immunostained for PrP with either the 6H4 antibody (a, b, c) or 3F4 (d, e, f) (green) and the nuclei counterstained with DAPI (blue). The scale bars (a) represent 20 µm.

3.5.3.17 Analysis of cells exposed to medium spiked with sCJD brain

To test whether the cells are equally able to take up PrP^{Sc} originating from other than acquired prion strain (iCJD, vCJD), the hESC and HK cells were exposed to sporadic CJD brain material.

The same immunocytochemical pre-treatment (PK and Gnd) and PrP^{Sc} detection with 3F4 or 6H4 antibody was used for analysis of cells exposed to sCJD brain material (Figure 3.45). Exposure of hESC of the RCM-1 line (A-F) and HK cells (G) to medium containing crude extracts of sCJD brain material of either of the most common subtypes MM1 (A, B, D, E, G) and VV2 (C, F) resulted in a quantitatively and morphologically similar immunostaining of internalised exogenous PrP^{Sc}. The typical perinuclear accumulation of PrP^{Sc} with a coarse granular morphology was observed in both cell types exposed to sCJD (A-G).

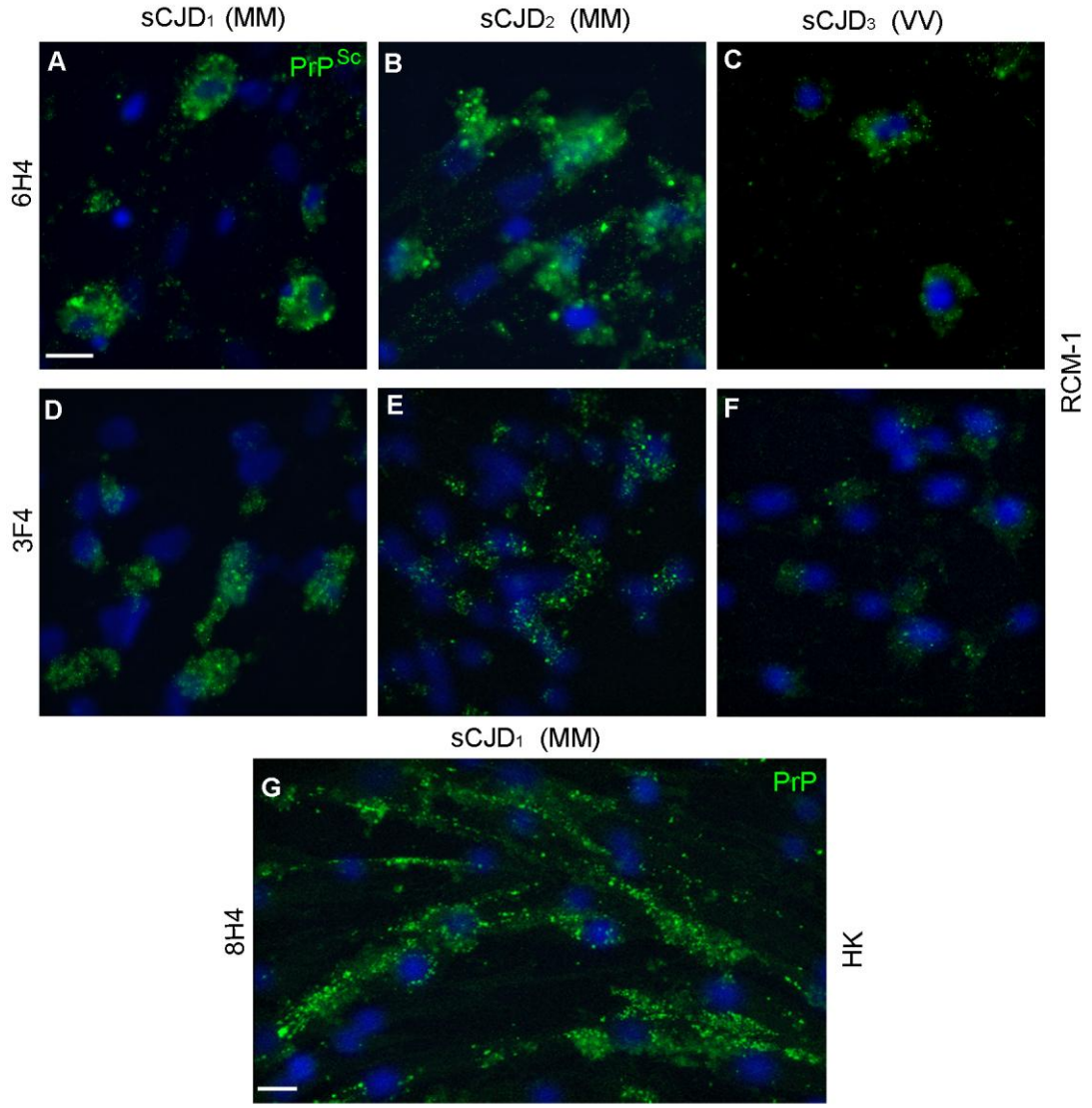


Figure 3.45: Exposure of cells to medium spiked with the sCJD (MM) and (VV)
PrP^{Sc} uptake from sporadic CJD (sCJD) brain by hESC and HK cells was analysed by immunocytochemistry. The method was featuring with PK/Gnd denaturation step (A-F). Briefly, the hESC of the RCM-1 cell line (A-F) and HK cell line (G) were exposed to $sCJD_1$ (MM1) subtype (A, D, G), $sCJD_2$ (MM1) subtype (B, E), or $sCJD_3$ VV2 subtype (C, F) 1% brain homogenate for 48 hours. Then the cells were fixed, permeabilised, treated with PK and Gnd and immunostained for PrP^{Sc} with the 6H4 antibody (A, B, C), or 3F4 antibody (D, E, F) or the 8H4 antibody (G) (green). The nuclei were counterstained with DAPI (blue) and the scale bars represent 20 µm.

3.5.3.18 Immunostaining for glial filaments in cells exposed to brain spiked medium

The data accumulated thus far raised the question of whether other proteins are taken up with PrP^{Sc} or whether the uptake mechanism was specific for PrP^{Sc}. To address this question (Figure 3.46), hESC of the RCM-1 cell line (A) and the FDC-like HK cells (B) were continuously exposed to vCJD (left column), AD (centre column) or cultured unexposed (right column) for 48 hours. The cells were then fixed, permeabilised, PK treated and double immunolabeled for PrP^{Sc} with 6H4 (green) (only in B) and glial filaments (glial fibrillary acidic protein, GFAP) (red) (A, B).

Immunostaining for glial filaments was observed in hESC cells exposed to vCJD (Aa) and to AD (Ab). Double immunolabeling in the HK cells (B) showed the same phenomenon. The merged channels show the typical juxtanuclear accumulation of PrP^{Sc} and GFAP material in vCJD exposed cells (Bg) and GFAP in AD exposed cells (Bh). This indicates that the material taken up by the hESC and HK cells is complex, including brain components other than PrP^{Sc}.

The PrP^{Sc} and GFAP uptake is representative of four independent, non-identical experiments. The differences involved type of cell line and the choice of antibodies used in the immunocytochemistry procedure (not all data are shown).

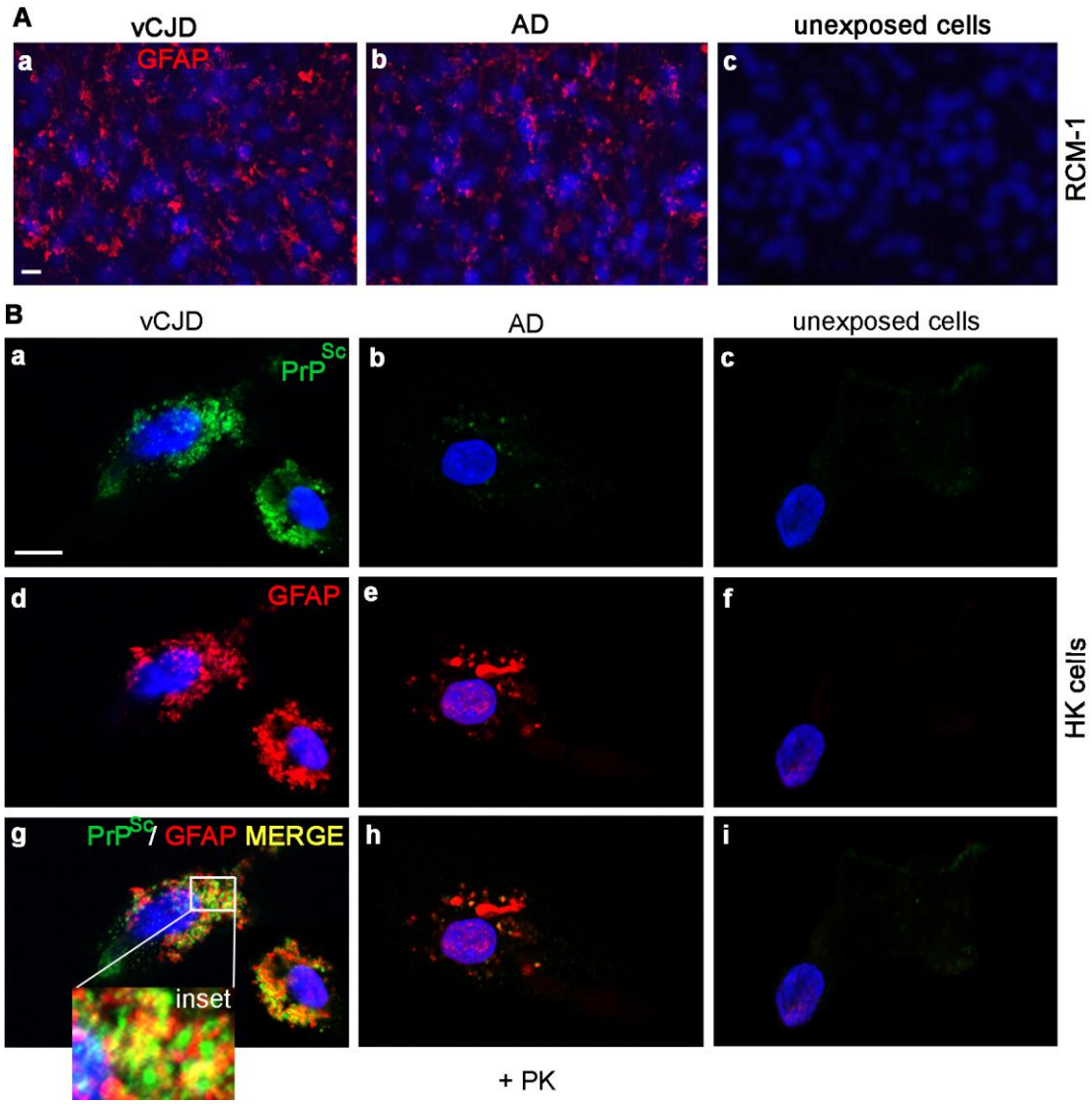


Figure 3.46: Immunostaining for glial filaments in cells exposed to brain spiked media

hESC of the RCM-1 cell line (A) and HK cells (B) were exposed to vCJD (Aa, B-left column), AD (Ab, B-middle column) 1% brain homogenate or grown in control medium 48 hours (Ac, B-right column). RCM-1 cells (A) were then fixed, permeabilised, treated with proteinase K and immunostained with an antibody for glial fibrillary acidic protein (GFAP, red). The HK cells (B) were fixed, permeabilised, treated with PK and double immunolabeled for PrP with the 6H4 antibody (Ba, Bb, Bc) (green) and GFAP (Bd, Be, Bf) (red) as described in section 2.12.4. Merge of the channels (PrP^{Sc}/GFAP) is shown in (Bg, Bh, Bi). Any colocalisation of PrP^{Sc} and GFAP appeared yellow. The nuclei were counterstained with DAPI (blue). The scale bars represent 20 µm.

3.5.3.19 Controls for the immunostaining procedures

The controls for the immunocytochemical procedures in both hESC (Figure 3.47; A-C) and HK (Figure 3.47; D-F) cell lines for antibody false positivity (A, B, D, E) or cellular auto-fluorescence (C, F) were negative and confirmed that all the previous results show genuine signals dependent on the specificity of the primary antibodies employed.

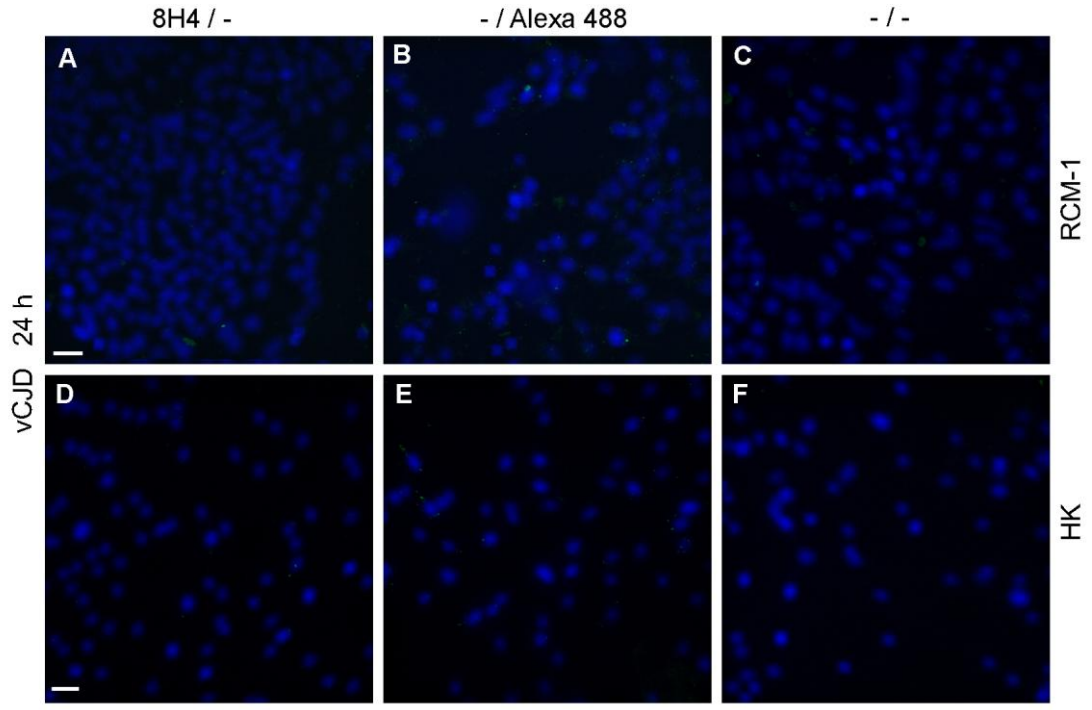


Figure 3.47 Controls for the immunostaining procedures
The hESC RCM-1 cell line (A-C) and HK cell line (D-F) were exposed to 1% vCJD brain spiked medium for 24 hours. Then cells were washed, fixed, permeabilised and immunostained omitting specific steps such as the FITC-labelled secondary antibody (A, D), the primary antibody 8H4 (B, E) and both primary and secondary antibodies (C, F). The nuclei were counterstained with DAPI (blue). Scale bar represents 50 µm.

3.5.4 Summary

- Undifferentiated hESC cells and FDC-like HK cells display a readily detectable time dependent uptake of PrP^{Sc} from medium spiked with prion disease brain homogenates.
- Cells challenged with infectious brain material showed strong intracellular accumulation of PrP^{Sc} with a course granular morphology.
- Morphological examination of cells exposed to prion spiked medium concluded that these cells did not suffer any observable damage that could be responsible for unusual localisation of PrP and the cells did not reveal any gross changes in cell morphology or viability caused by PrP^{Sc} uptake and accumulation in the cytosol.
- No differences in the uptake were observed which could be attributed to differences of cell type (FDC-like vs. stem cells).
- It was concluded that the uptake is neither dependent on the species origin of the brain homogenate nor the *PRNP* codon 129 genotype of the cell. The intense intracellular staining of exogenous PrP^{Sc} within cells exposed to BSE, vCJD, iCJD, sCJD further confirmed previous observations that PrP^{Sc} uptake by cells at each given time point showed no observed differences amongst the different prion agents.
- The live cell staining resulted in no PrP^{Sc} immunostaining, indicating the positive immunostaining is material accumulated intracellularly, rather than resulting from brain material deposited on the external surface of the cells.

- Immunostaining for glial filaments in exposed and unexposed cells indicated that the material taken up by cells is complex, and includes brain components other than PrP^{Sc}.

3.6 KINETICS OF EXOGENOUS PrP^{Sc} UPTAKE AND CLEARANCE ANALYSED BY IMMUNOCYTOCHEMISTRY

3.6.1 Objectives

To examine the kinetics of exogenous PrP^{Sc} taken up by human embryonic stem cells and the FDC-like HK cells.

3.6.2 Rationale

Following on from the previous findings (section 3.3-4), - that exogenous PrP^{Sc}, once taken up, was subsequently lost when the HK cells were allowed to grow in control medium, - it was of interest to examine whether the human embryonic stem cells exposed to infectious prion material behave similarly. It was shown earlier (section 3.5.4) that these cells (RCM-1) are able to rapidly take up prion material. Whether the human embryonic stem cells are also able to clear this material as HK cells do, or whether prion infection becomes established remains to be demonstrated. These data do provide a valuable insight into the potential for prion propagation in undifferentiated stem cells. The comparison of the PrP^{Sc} clearance in human lymphoreticular FDC-like cells with human undifferentiated stem cells may also provide important insights into the cell biology of these two cultured cell types. Based on previous experience, immunocytochemistry was chosen as the best tool to employ in this investigation.

The PrP^{Sc} uptake and clearance by HK cells and hESC analysed by ICC is representative of two independent, non-identical experiments.

3.6.3 Experimental results

3.6.3.1 Clearance of PrP^{Sc} from exposed hESC analysed by immunocytochemistry

To determine the kinetics of PrP^{Sc} clearance in hESC, we exposed the cells of the RCM-1 line (Figure 3.48) to BSE (A, E, I, M, R), vCJD (B, F, J, N, S) and AD (C, G, K, O, T) 1% brain spiked medium, or cultured cells in control medium (D, H, L, P, U). The immunostaining protocol, which included Gnd pre-treatment, was used to diminish the PrP^{C} and enhance the detection of PrP^{Sc} immunostaining in the analysed samples. After 48 hours of continuous exposure the PrP^{Sc} immunostaining signal indicated an intracellular localisation in hESC exposed to infectious prion material (A, B). The medium was then withdrawn (and retained for further analysis) and cells were cultured in fresh (unspiked) medium for 24 hours.

The PrP^{Sc} signal in BSE and vCJD exposed cells was observed to have fallen considerably (E, F). The signal was further reduced after another change of fresh control medium followed by a further 24 hours incubation (48 hours recovery) (I, J). By 72 hours of recovery and three changes of control medium, only low or background staining was present in the majority of cells exposed to BSE and vCJD (M, N). However, above background levels of PrP^{Sc} could be still observed in less common clusters of cells in the 72 hours' recovery time point (R, S). The staining pattern of the cell-associated PrP^{Sc} changed over the time of recovery from a bright perinuclear PrP^{Sc} "cloud" to discrete dot like appearance. Cells exposed to AD brain homogenate remained negative for PrP^{Sc} immunostaining throughout the experiment (C, G, K, O, T), as did the control unexposed cells (D, H, L, P, U).

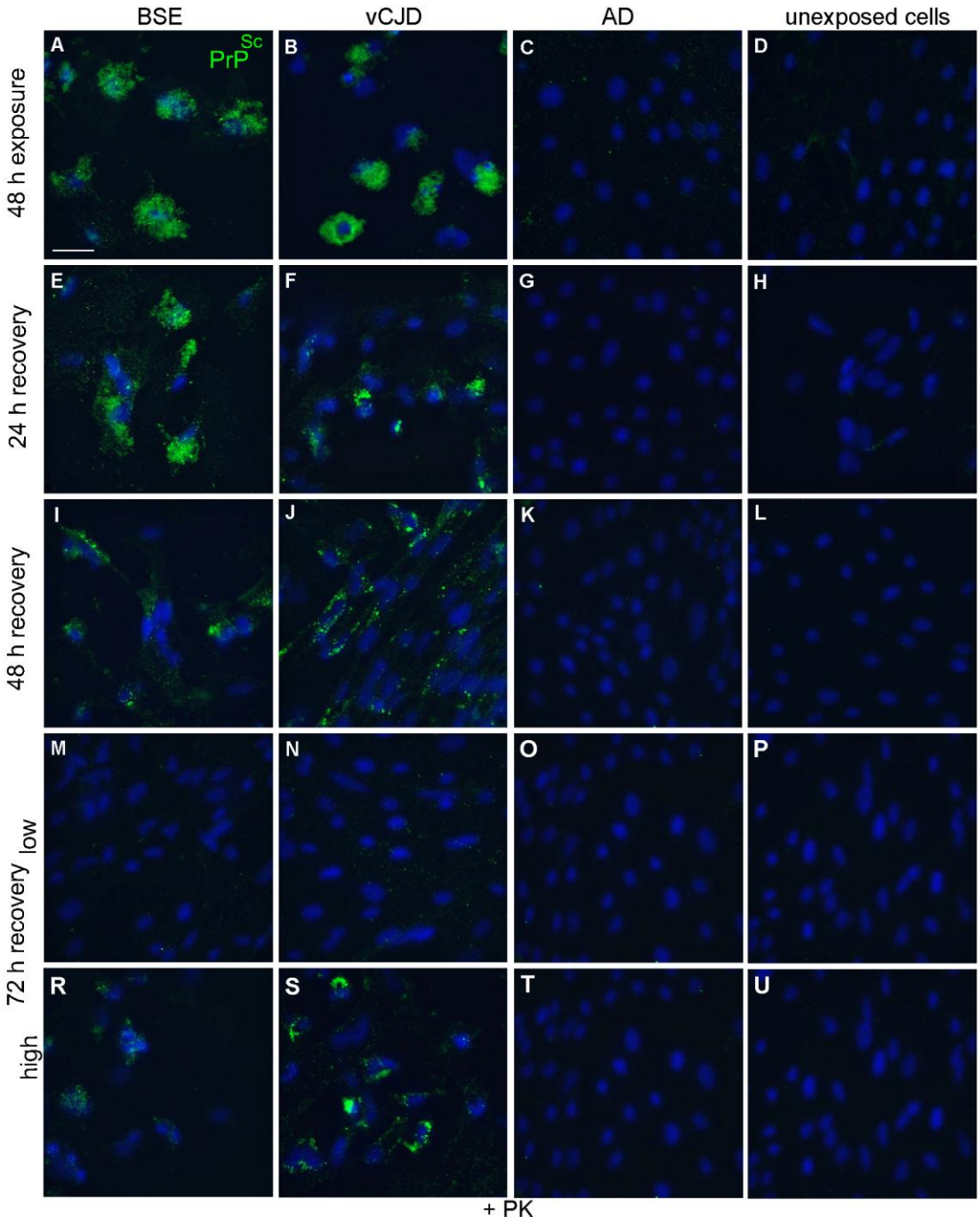


Figure 3.48: Clearance of PrP^{Sc} from exposed hESC analysed by immunocytochemistry
The RCM-1 cells were exposed to BSE (A, E, I, M, R), vCJD (B, F, J, N, S), AD (C, G, K, O, T) 1% brain homogenate or grown in a control medium (D, H, L, P, U) for 48 hours (A-D). The medium was then withdrawn, the cells were washed and given fresh control medium and allowed to continue growing for a further 24 hours (E-H), 48 hours (I-L) or 72 hours (M-U) with medium changes every 24 hours. The cells were then washed, fixed, permeabilised, pre-treated with PK and immunostained for PrP using the antibody 6H4 (green) and the nuclei counterstained with DAPI (blue). The fields shown in (M-P) are typical of the cultures observed at the 72 hours recovery time point, whereas less common clusters of cells immunostaining for PrP^{Sc} are shown in (R, S). The scale bar represents 50 µm.

3.6.3.2 Western blot analysis of PrP^{Sc} in culture medium incubated with RCM-1 cells during exposure and recovery

To determine whether the loss of the PrP^{Sc} immunostaining signal from the hESC cells was due to an effective exocytosis, a Western blot analysis of the retained medium incubated with cells was performed (Figure 3.49). Analysis of BSE (A) and vCJD (B) PrP^{Sc} before (lane 1) and after 48 hours of incubation with the cells (A and B, lane 2) showed an apparent loss of the PrP^{Sc} from the media. At this same time point PrP^{Sc} could be detected intracellularly, as analysed by ICC (Figure 3.48; A and B). Figure 3.49; lane 3, 4, 5 corresponded to the 24 hours recovery intervals of fresh media incubated with the exposed cells, yet show no signs of detectable amounts of the cell-associated PrP^{Sc} shed back into the medium.

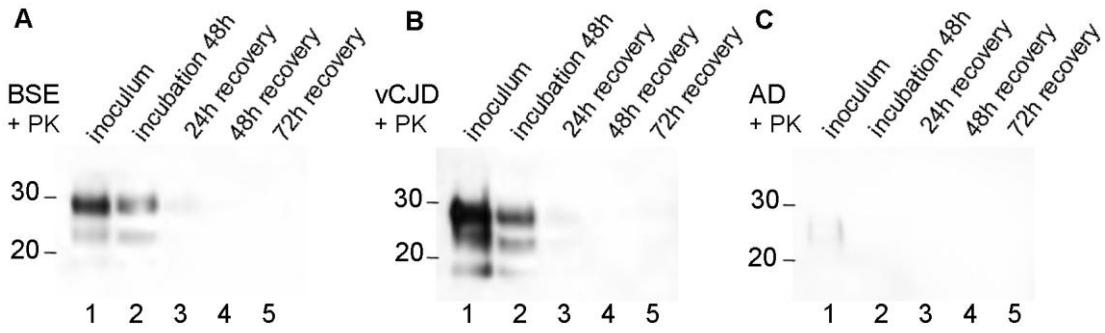


Figure 3.49: Western blot analysis of PrP^{Sc} in culture medium incubated with RCM-1 cells during exposure and recovery

The hESC RCM-1 cells were exposed to 1% BSE (A), vCJD (B) or AD (C) spiked medium for 48 hours. After incubation the medium was withdrawn and retained for Western blot analysis. The process was repeated also at every 24 hours recovery time point, up to the total 72 hours recovery time period. Equivalent volumes of the cell culture medium (incubated with the cells during the experiment) were digested with PK and analysed by Western blotting for PrP^{Sc} presence using the antibody 6H4. The culture media analysed were: the brain input into the culture medium prior to incubation with the cells (lane 1), the same culture medium after 48 hours of incubation with the cells (lane 2), fresh medium after a 24 hours of incubation (24 hours recovery) (lane 3), the second change of fresh medium after a further 24 hours of incubation (48 hours recovery) (lane 4), and the third change of fresh medium after a further 24 hours of incubation (72 hours recovery) (lane 5). The molecular weight, in kDa, is marked left on each blot.

Interestingly, the loss of the PrP^{Sc} immunostaining signal from the cells was rapid (Figure 3.48; BSE - I, J, M, R; vCJD - F, J, N, S) and it is unlikely that the material could be simply diluted out by cell division given that the typical doubling time of these hESC is around 35 hours (Beattie and Hayek, 2004; Gearhart, 2004).

3.6.3.3 Clearance of PrP^{Sc} from exposed HK cells monitored up to 72 hours recovery analysed by immunocytochemistry

The immunocytochemical visualisation of the kinetics of PrP^{Sc} clearance in the human FDC-like HK cells is shown in Figure 3.50. HK cells were continuously exposed to BSE (A, E, I, M), vCJD (B, F, J, N), AD (C, G, K, O) 1% brain homogenate or grown in control medium (D, H, L, P). The immunostaining protocol including a Gnd pre-treatment was used to enhance the PrP^{Sc} signal. The time of exposure was 48 hours (A-D). The PrP^{Sc} immunosignal in cells exposed to BSE (A) and vCJD (B) could be observed intracellularly. Then the brain spiked medium was removed and cells were allowed to grow in control (unspiked) medium up to 72 hours with medium changes at 24 hours intervals. The kinetics of PrP^{Sc} clearance examined after the 24 hours recovery (E-H), 48 hours recovery (I-L) and 72 hours recovery (M-P) in BSE (E, I, M) and vCJD (F, J, N) exposed HK cells appeared to be slower in these cells when compared to the hESC (Figure 3.48). The typical PrP^{Sc} immunostaining signal represented by coarse granular morphology in the cells exposed to the crude BSE or vCJD brain homogenates (A, B) could be observed in the cytosol of the cells within the whole course of the recovery period (72 hours). Therefore the clearance was considered to be less effective by the HK cells when compared to the hESC.

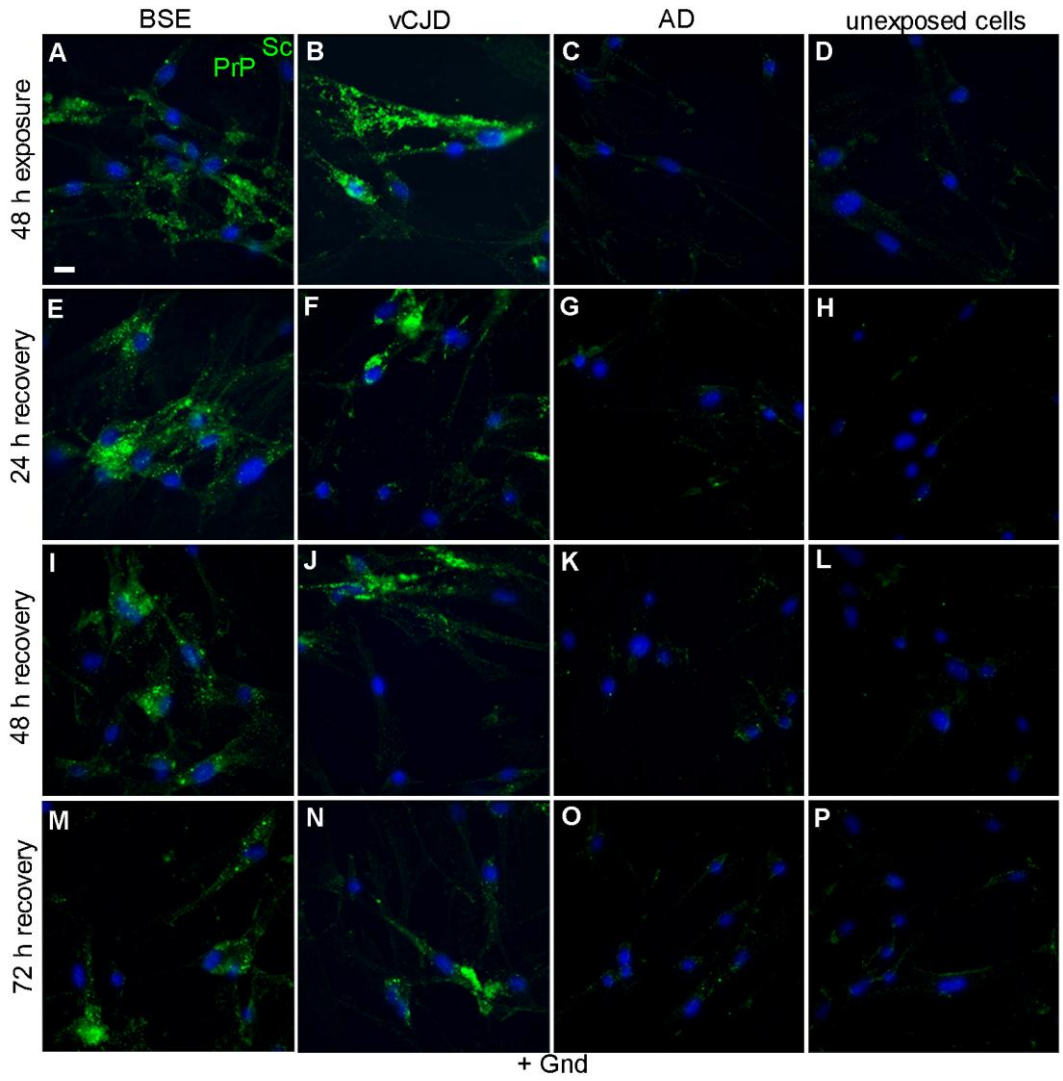


Figure 3.50: Clearance of PrP^{Sc} from exposed HK cells monitored up to 72 hours recovery analysed by immunocytochemistry

The HK cells were exposed to BSE (A, E, I, M), vCJD (B, F, J, N), AD (C, G, K, O) 1% brain homogenate or grown in a control medium (D, H, L, P) for 48 hours (A-D). The medium was then withdrawn, the cells were washed and given fresh control medium and allowed to continue growing for a further 24 hours (E-H), 48 hours (I-L) or 72 hours (M-P) with medium changes at 24 hours intervals. The cells were then washed, fixed, permeabilised, pre-treated with Gnd and immunostained for PrP using the antibody 6H4 (green). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.6.3.4 Western blot analysis of PrP^{Sc} in culture medium incubated with HK cells during exposure and recovery

The media incubated with the HK cells in the experiment described above were also retained and analysed by Western blot for PrP^{Sc} presence (Figure 3.51). The input of

BSE (A), vCJD (B) and AD (C) is shown in lane 1. PrP^{Sc} uptake is indicated by the loss of the signal from media after 48 hours incubation (lane 2) with the HK cells (appeared as an intracellular signal, Figure 3.50; A and B). The medium was changed at 24-hour intervals (lane 3, 4, 5), representing 72 hours of recovery in total. The analysis showed no signs of detectable PrP^{Sc} shed back to the medium. It is important to note that the PrP^{Sc} clearance in HK cells appears to be a slow process and a longer recovery time was required to draw firm conclusions about the clearance rate (see following section 3.6.3.5).

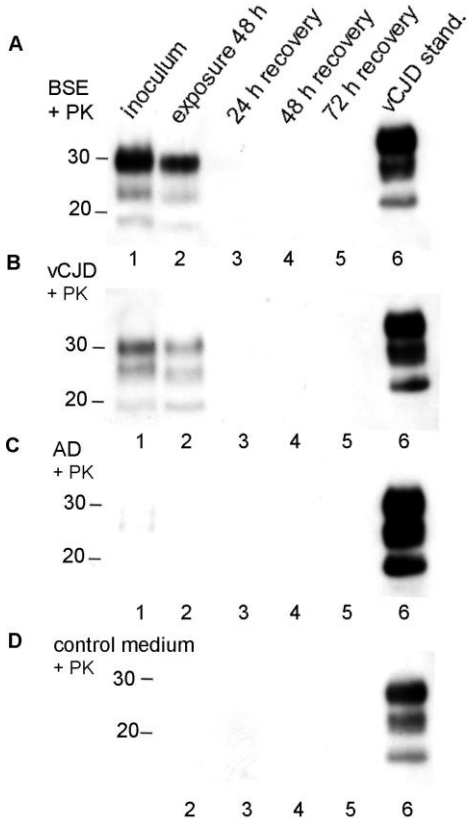


Figure 3.51: Western blot analysis of PrP^{Sc} in culture medium incubated with HK cells during exposure and recovery

The medium incubated with HK cells exposed to 1% BSE (A), vCJD (B), AD (C) spiked medium or grown in control medium (D) for 48 hours and during the recovery time was retained. Equivalent volumes of the medium were digested with PK and analysed by Western blotting using the antibody 6H4. The culture media analysed were: the brain input into the culture medium prior to incubation with the cells (lane 1), the same culture medium after 48 hours of incubation with the cells (lane 2), a fresh medium after a 24 hours of

incubation (24 hours recovery) (lane 3), the second change of fresh medium after a further 24 hours of incubation (48 hours recovery) (lane 4), and the third change of fresh medium after a further 24 hours of incubation (72 hours recovery) (lane 5). The vCJD 2B standard (lane 6) was used as a control of PrP migration and blotting procedure. The molecular weight, in kDa, is marked left on each blot.

3.6.3.5 Clearance of PrP^{Sc} from the exposed HK cells monitored at long term recovery analysed by immunocytochemistry

Based on the observations from the study above, a further experiment examining the kinetics of PrP^{Sc} clearance in HK cells at longer term of recovery was undertaken (Figure 3.52). The optimised immunostaining protocol (including Gnd pre-treatment) was used to examine the kinetics of PrP^{Sc} clearance. HK cells were exposed to 1% $iCJD_1$ brain spiked medium for 48 hours (A). The switch from BSE or vCJD to iCJD was performed in an attempt to visualise the previously observed clearance of PrP^{Sc} from HK cells analysed by Western blot (section 3.4). PrP^{Sc} immunostaining resulting from $iCJD_1$ gave a quantitatively weaker signal when compared to BSE and vCJD (Figure 3.50; A, B) exposed cells. In this assay the exposed cells were given a 96 hours recovery period with fresh medium changes at 24-hour intervals (B, C, D, E) and again only slight PrP^{Sc} clearance could be observed even at this slightly longer recovery time. This was followed by a subsequent culturing including three passages (every 7 days) (F, G, H). Gradual and progressive loss of PrP^{Sc} immunostaining signal from HK cells could be observed with subsequent passaging. The clearance of PrP^{Sc} immunostaining signal first represented by the typical coarse granular morphology in the cells exposed to the crude iCJD brain homogenate (A) progressed to a finer patchy signal after 1^{st} passage (F) and was lost from the cells after the 3^{rd} passage (H).

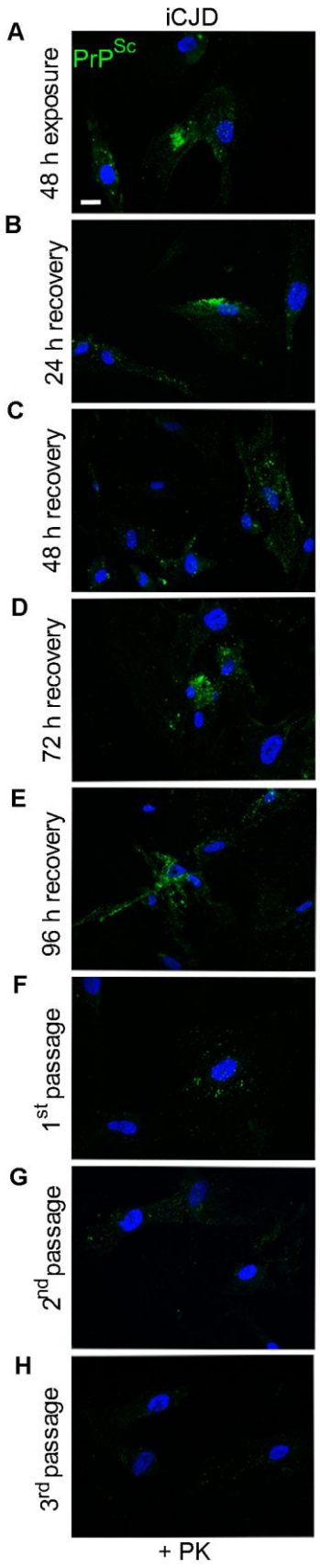


Figure 3.52: Clearance of PrP^{Sc} from exposed HK cells monitored at longer term recovery analysed by immunocytochemistry

HK cells were continuously exposed to 1% $iCJD_1$ brain spiked medium for 48 hours (A). The medium was then withdrawn, the cells were washed and given fresh medium (without brain homogenate) and allowed to continue growing for a further 24 hours (B), 48 hours (C), 72 hours (D), or 96 hours (E) with medium changes at 24 hours intervals. After reaching 70-80% confluence, the cells were split in a manner where a small fraction of cells was plated onto a chamber slide for the ICC analysis (F) and the rest of the cells were plated into fresh T25 cell culture flask and allowed to grow until confluent. This passaging process was then repeated twice (G, H). The immunostaining procedure was routinely performed. In short, the cells were washed, fixed, permeabilised, pre-treated with PK and immunostained for PrP using the antibody 6H4 (green) and the nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.6.4 Summary

- Judging by the sensitive immunostaining of the PrP^{Sc} in exposed cells and the loss of the protease-resistant prion protein from the brain spiked medium, as shown by Western blots, the hESC and HK cells appear to have mechanisms that allow a rapid uptake of brain material (including a disease-associated and most likely infectious form of the prion protein).
- Based on findings from this recovery assay, these cells are equally able to clear PrP^{Sc} at a rapid rate, suggesting that they also have an efficient mechanism to presumably degrade, or otherwise process the material taken up.
- Interestingly, the clearance of infectious prions from the human FDC-like HK cells appeared to be a slower process when compared to the hESC.

3.7 KINETICS AND MECHANISM OF ENDOCYTOSIS OF EXOGENOUS PrP^{Sc} IN HK CELLS ANALYSED BY IMMUNOCYTOCHEMISTRY

3.7.1 Objectives

To determine the time dependent mechanism of internalisation by which exogenous PrP^{Sc} is taken up from brain spiked media by extraneuronal lymphoreticular FDC-like HK cells.

3.7.2 Rationale

The mechanism of PrP^{Sc} internalisation, trafficking and the cellular site of conversion remain unresolved questions in prion disease pathogenesis. The available evidence suggests a variety of routes and these may be influenced by the cell type under examination and other factors specific to the experimental design. Endocytosis is a basic cellular process that is used by cells to internalise a variety of molecules. Identification of the mechanism by which PrP^{Sc} enters the cell is a vital aspect in understanding the prion infection mechanism. Based on the results from sections 3.4-6, the PrP^{Sc} from brain spiked media does indeed enter the exposed cells and the mechanism of this process was investigated here by examining the extraneuronal lymhoreticular FDC-like HK cells when acutely exposed to prions.

3.7.3 Experimental results

3.7.3.1 ICC of caveolae-coated vesicles and clathrin-coated pits in HK cells

To clarify the routes of internalisation of the exogenous PrP^{Sc} into the HK cells, a series of experiments were designed, concentrating on the two main elements of the endocytic mechanisms, i.e. the caveolae-coated vesicles (Figure 3.53; A) and

clathrin-coated pits (Figure 3.53; B). Caveolae-coated vesicles were densely represented at the HK cell plasma membrane (A-red), as were the invaginations of the clathrin coated pits (B-red).

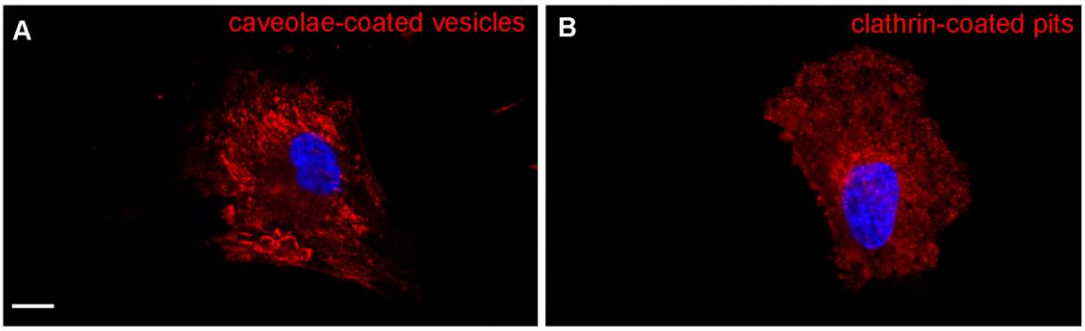


Figure 3.53: Immunostaining of caveolae-coated vesicles and clathrin-coated pits in HK cells
HK cells were washed, fixed, permeabilised and immunostained for caveolae-coated vesicles (A) with antibody caveolin1 and clathrin coated pits (B) with clathrin, these were revealed by incubation with Alexa 546 conjugated secondary antibody for 60 minutes as described in section 2.12.4. The nuclei were counterstained with DAPI (blue). The scale bar, 20 µm.

3.7.3.2 Double immunolabeling of PrP^{Sc} and caveolae-coated vesicles after continuous exposure of HK cells to vCJD brain spiked medium

In order to determine the possible involvement of caveolae-coated vesicles in the uptake of PrP^{Sc} from brain spiked media, the HK cells were incubated with medium containing sonicated 1% vCJD brain homogenate for 2, 4 and 48 hours. Unexposed control cells were incubated with unspiked medium. The cells were analysed by confocal microscopy after being double immunolabeled for PrP with primary antibody 8H4 (green) and caveolae-coated vesicles with anti-caveolin1 (red) (Figure 3.54). The merging of the channels is shown and any colocalisation of PrP with caveolae-coated vesicles appears in yellow.

The 2-hour time point of exposure to vCJD spiked medium (B) appeared to have a same amount of PrP (green) as the control cells (A) and no positive colocalisation of

exogenous PrP^{Sc} and caveolae-coated vesicles could be observed at this early point. However, the time points analysed at 4 (C) and 48 hours (D) showed some evidence of positive colocalisation of exogenous PrP^{Sc} with caveolae-coated vesicles (yellow dots and clumps). Therefore, from the 4-hour and 48-hour time points it could be presumed that caveolae-coated vesicles of HK cells might be implicated in the PrP^{Sc} uptake. It must be noted that the incubation with brain spiked medium in this case was continuous, like in all previous experiments. Ideally, a reconstruction of the principle for future experiments investigating the internalisation and trafficking mechanisms of PrP^{Sc} was needed. Having considered the objective purpose of this investigation, a different approach for this experiment was therefore formatted.

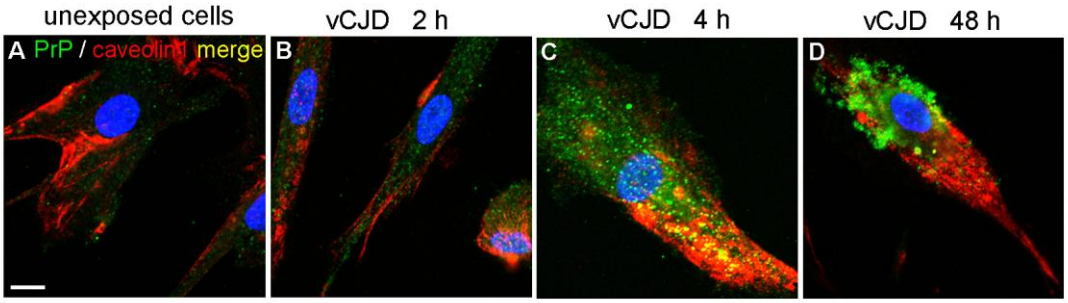


Figure 3.54: Double immunolabeling of PrP^{Sc} and caveolae-coated vesicles after continuous exposure of HK cells to vCJD brain spiked medium
HK cells were continuously exposed to medium containing 1% vCJD brain homogenate for 2 (B), 4 (C) and 48 hours (D), or grown in a control medium (A). The cells were then washed, fixed, permeabilised and double labelled for PrP with antibody 8H4 (green) and caveolae-coated vesicles with caveolin1 antibody (red). The nuclei were counterstained with DAPI (blue). Shown is the merge of all channels. The scale bar represents 20 µm.

In the new approach the so called "pulse and chase" principle was applied. This approach - for mapping prion uptake and its trafficking over time - was designed to provide a better definition of the processes involved. It avoids potential complications of material continually entering the trafficking system and obscuring

the processing of previously taken up materials. The principle of a "pulse and chase" experiment is described in schematic form in section 2.11.2.3.

Briefly, cells were exposed to medium containing 6H4 (either control or brain spiked) for short time period, 30 minutes at 4 °C and additional 15 minutes at 37 °C, to initiate the internalisation process – the "pulse". Then the cells were extensively washed to remove any material deposited on the cell surface and further cultured in control medium for desired time period – the "chase". The cells were then immunostained at different time points of the "chasing" phase. It should be noted that the incubation with anti-prion primary antibody is therefore no longer needed in the immunostaining protocol. The fixed and permeabilised cells were blocked and subsequently incubated with the FITC secondary antibody Alexa 488 labelling the PrP-6H4 complexes taken up during the "pulse" phase. An immunocytochemical pre-treatment with guanidine (enhancing PrP^{Sc} and diminishing PrP^{C} signal) was used when needed to distinguish exogenous PrP^{Sc} from the cellular PrP^{C}. Subsequently, the samples were washed and immunostained with caveolin1 or clathrin antibodies overnight, which were revealed by incubation with Alexa 546 for 60 minutes. The nuclei were counterstained with DAPI and slides were then mounted with mounting media and examined by confocal microscopy.

3.7.3.3 Preliminary "pulse and chase" experiment (3 hours "chase" period)

The preliminary "pulse and chase" experiment was designed with the "chasing" period consisting of 3 hours. This was primarily aimed to identify any PrP signal differences of the unexposed and exposed cells to brain spiked medium (showing exogenous PrP^{Sc} to be cell-associated), determining that the "pulse" time period was long enough for cells to initialise internalisation of the antibody-antigen (6H4-vCJD

PrP^{Sc}) complex. This also allows to observe whether the 3 hours "chase" period would reveal which mechanism of internalisation was predominantly implicated in the early time point of PrP uptake and which in the latter (Figure 3.55). The HK cells were then immunolabeled for caveolae coated vesicles with caveolin1 (Figure 3.55; A and B) or clathrin-coated pits with clathrin (Figure 3.55; C and D).

A clear difference could be observed in the PrP signal (green) of the cells incubated with unspiked medium (A, C) when compared to the vCJD exposed cells (B, D), indicating that the individual time settings of the "pulse and chase" experiment were sufficient. However, the most interesting observation was the positive colocalisation (yellow) of PrP^{Sc} with caveolae-coated vesicles at 3 hours chase period (B), but no detectable evidence of PrP^{Sc} colocalisation with clathrin-coated pits (D) at this time period. This suggests that additional investigation of earlier and latter time points might reveal a possible time dependent involvement of those two uptake mechanisms or the alternative possibility that the clathrin-coated pits may not be involved in exogenous PrP^{Sc} endocytosis at all in the HK cells.

The difference in the PrP (green) signal of unexposed cells (A, C) compared to the PrP signal of the cells exposed to brain spiked medium (B, D) indicates that the vCJD PrP-6H4 is taken up together as a complex, rather than the 6H4 primary antibody being taken up on its own. The results show that this was not the case, and the colocalisation of exogenous PrP^{Sc} with endocytic vesicles implicated in the internalisation process was convincingly demonstrated. Nevertheless, the observed very faint sign of colocalisation (yellow) in unexposed cells (A) could indicate that the HK cellular PrP^{C} is being recycled via caveolae-coated vesicles in these cells.

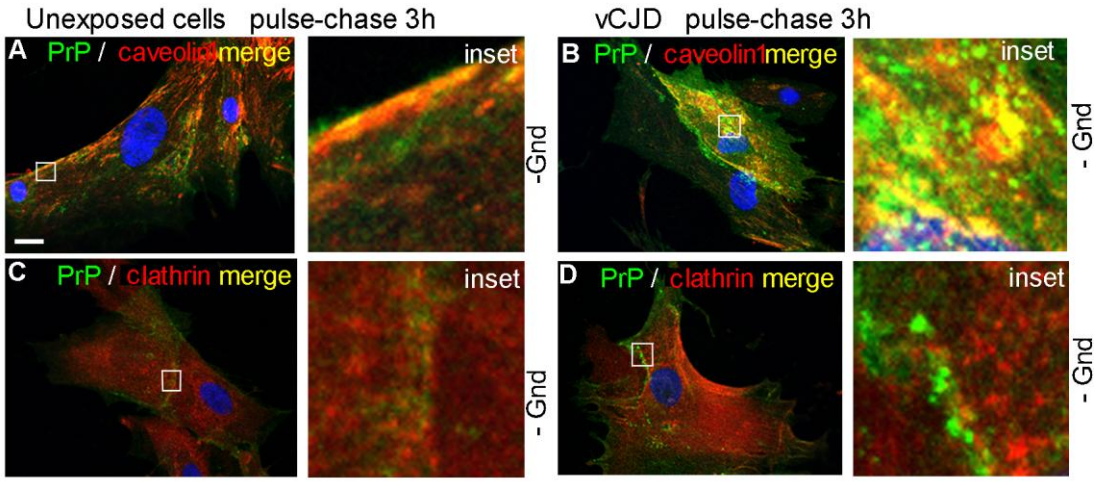


Figure 3.55: Preliminary "pulse and chase" experiment (3 hours "chase" period)
HK cells were incubated with either control medium (A, C) or 1% vCJD spiked medium (B, D) already containing anti-PrP primary antibody 6H4 (1 µg/ml) for 30 minutes at 4 °C – "pulse". Then the cells were washed and fresh control medium was added. Cells were subsequently placed at 37 °C for 3 hours to initiate the internalisation of the antigen-antibody complex (vCJD/PrP-6H4) – "chase". The cells were then fixed, permeabilised, blocked and incubated with Alexa 488 labelling PrP-6H4 complexes (green) for 60 minutes. Subsequently the samples were immunostained for caveolae-coated vesicles (A, B) with caveolin1 antibody or for clathrin coated pits (C, D) with clathrin antibody which were revealed by incubation with Alexa 546 (red) as described in section 2.12.5. Positive colocalisation of PrP (green) and endocytic vesicles (red) appeared yellow. Insets represent magnification of the boxed areas. The nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.7.3.4 "Pulse and chase" experiment - (24 hours "chase" period)

Following the pilot "pulse and chase" study, a further experiment was conducted to determine the involvement of these two different elements of the endocytic machinery at a later time point. A "pulse and chase" experiment was performed using a 24-hour "chasing" time period (Figure 3.56). The HK cells exposed in "pulse" to 6H4-vCJD brain spiked medium and a 24-hour "chase" were double immunolabeled for caveolae-coated vesicles (A, B) or clathrin-coated pits (C, D) (red) and prion protein (green). During the immunostaining, the cells were either guanidine pre-treated (B, D) to accentuate PrP^{Sc} or left untreated (A, C). A positive

colocalisation (yellow) of the exogenous PrP^{Sc} taken up and "chased" for 24 hours through the HK cells "pulsed" with vCJD brain spiked medium was clearly shown to be colocalised with caveolae-coated vesicles at this time point. The observation was evident in cells not pre-treated with guanidine (A) and also in cells pre-treated with guanidine in which case the cells own PrP^{C} was diminished and the exogenous PrP^{Sc} staining signal was enhanced (B). At this time point, a positive colocalisation of exogenous PrP^{Sc} with clathrin-coated pits was also observed (yellow patches) in cells either pre-treated with guanidine (D) or left untreated (C).

The outcome of this experiment indicates that the caveolae-coated endocytic machinery may be still involved in prion internalisation even after 24 hours post exposure (A and B) (as shown by positive colocalisation of PrP^{Sc} with caveolin1 in yellow). But more interestingly, the clathrin-coated pits also seem to be involved in the prion internalisation at this time point (C and D).

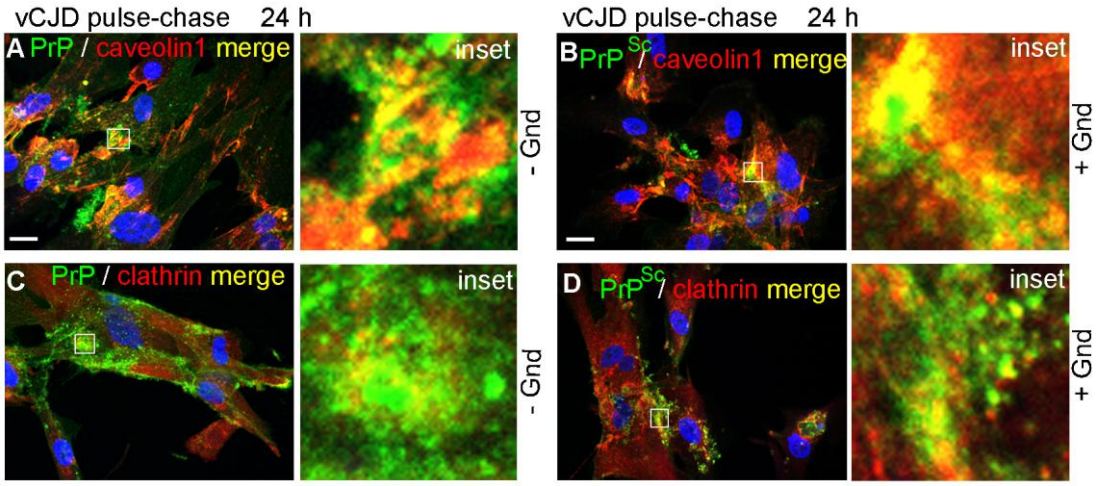


Figure 3.56: "Pulse and chase" experiment - (24 hours "chase" period)
HK cells were incubated with 1% vCJD brain spiked medium containing antibody 6H4 (1 µg/ml) for 30 minutes at 4 °C. Then the cells were placed at 37 °C for additional 15 minutes to initiate the internalisation of the antigen/antibody complex – "pulse". Subsequently, the cells were extensively washed with HBSS solution. Fresh control medium was added to the cultures and the cells were then cultured at 37 °C for 24 hours – "chase". The medium was then aspirated, the cells were extensively washed and double immunolabeled for PrP (green) and endocytic vesicles (red) as marked on the figure and previously described in section 2.12.5. Merge of the channels is shown. Insets represent magnification of the boxed areas. The double labelling and colocalisation (yellow) for PrP (A, C), or PrP^{Sc} after guanidine pre-treatment (B, D) with caveolae coated vesicles is shown in the top row (A, B) and for clathrin coated pits in the bottom row (C, D). The nuclei were counterstained with DAPI (blue). The scale bars, 20 µm.

In order to quantitatively define the respective involvement of caveolae and clathrin mediated endocytosis in the PrP^{Sc} uptake, more elaborate designs of the "pulse and chase" assays were performed (Figure 3.57-61). The aim was to examine time-dependent changes in the involvement of the two endocytic pathways and better distinguish PrP^{Sc} when using guanidine pre-treatment.

3.7.3.5 PrP^{Sc} colocalisation with caveolae coated vesicles in "pulse and chase" study (I.)

Firstly, the involvement of caveolae-coated vesicles in the endocytosis of the exogenous PrP^{Sc}, without guanidine pre-treatment during immunolabeling, was investigated (Figure 3.57). HK cells were "pulsed" with medium containing 6H4 primary antibody – either control (Figure 3.58; A-D) or vCJD spiked (Figure 3.58; E-H, insets of boxed areas). The cells were then double immunolabeled for PrP (green) and caveolae-coated vesicles (red) at 1 (A, E), 3 (B, F), 24 (C, G) and 48 hours (D, H) of the "chasing" time period as described in section 2.12.5. The merge of the channels is shown. Positive colocalisation of prion proteins and caveolae-coated vesicles appears in yellow.

The exogenous PrP^{Sc} was clearly demonstrated to be colocalised with caveolae-coated vesicles at 1 (E), 3 (F), and 24 hours (G) of the "chasing" period in HK cells exposed to vCJD spiked medium. This could be observed as yellow patches and clusters. The 48-hour time point also showed a positive colocalisation, although the signal of exogenous PrP^{Sc} appeared to be slightly weaker (H). The difference in the PrP signal (duller green) of unexposed cells (A-D) compared to the PrP signal of the cells exposed to brain spiked medium (intense green) (E-H) indicated, that the vCJD/PrP-6H4 was efficiently taken up together as a complex. This allowed us to clearly distinguish between the exogenous PrP^{Sc} (intense green, E-H, insets) taken up by cells exposed to brain material from the recycling cell own PrP^{C} (duller green, A-D). The results convincingly demonstrated that the caveolae-coated endocytic vesicles were clearly implicated in the internalisation process of exogenous PrP^{Sc}.

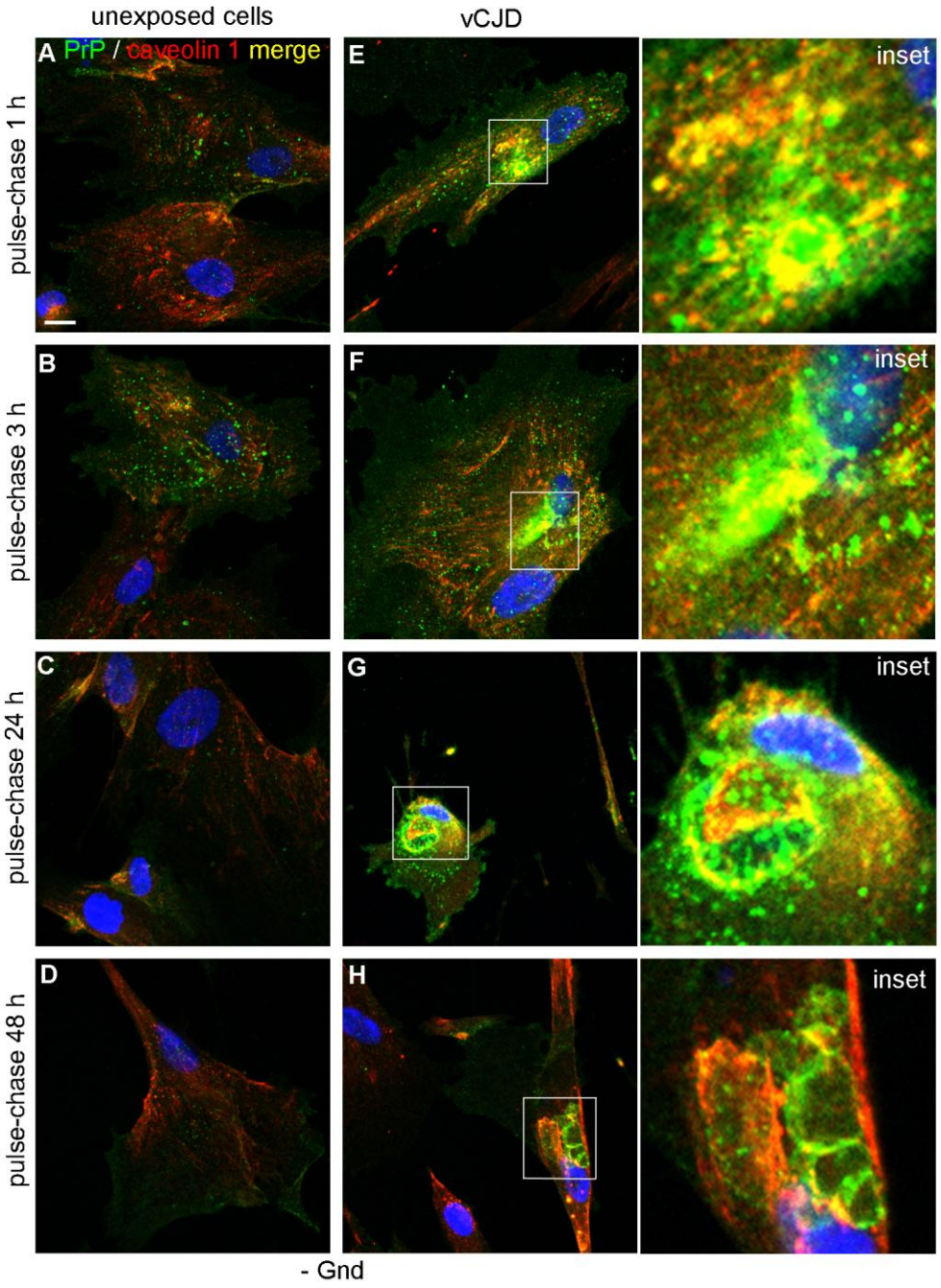


Figure 3.57: PrP^{Sc} colocalisation with caveolae coated vesicles in "pulse and chase" study (I.)

HK cells were incubated with medium containing 6H4 (1 µg/ml), either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". The cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and the cells were double immunolabeled for PrP (green) and caveolae coated vesicles (red) as was previously described in section 2.12.5. The merge figures of the channels are shown, any colocalisation appeared as yellow colour and the high magnifications of the boxed areas in (E-H) are shown right (inset). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.7.3.6 PrP^{Sc} colocalisation with caveolae coated vesicles in a "pulse and chase" study (II.)

More convincing endocytic parameters for PrP^{Sc} internalisation could be observed by using guanidine pre-treatment in immunostaining protocol (Figure 3.58) in the same experimental design as shown in previous investigation (section 3.7.3.5). Positive colocalisation of the exogenous PrP^{Sc} (green) (E-H, inset of boxed areas) with caveolae-coated vesicles (red) at 1-24 hours post exposure was observed (yellow). The PrP^{Sc} signal at the 48 hours "chasing" period (H) was not convincingly demonstrated to colocalise with caveolae-coated vesicles. As expected, after guanidine pre-treatment, the PrP^{C} signal was reduced when compared to untreated cells in the previous experiment (section 3.7.3.5).

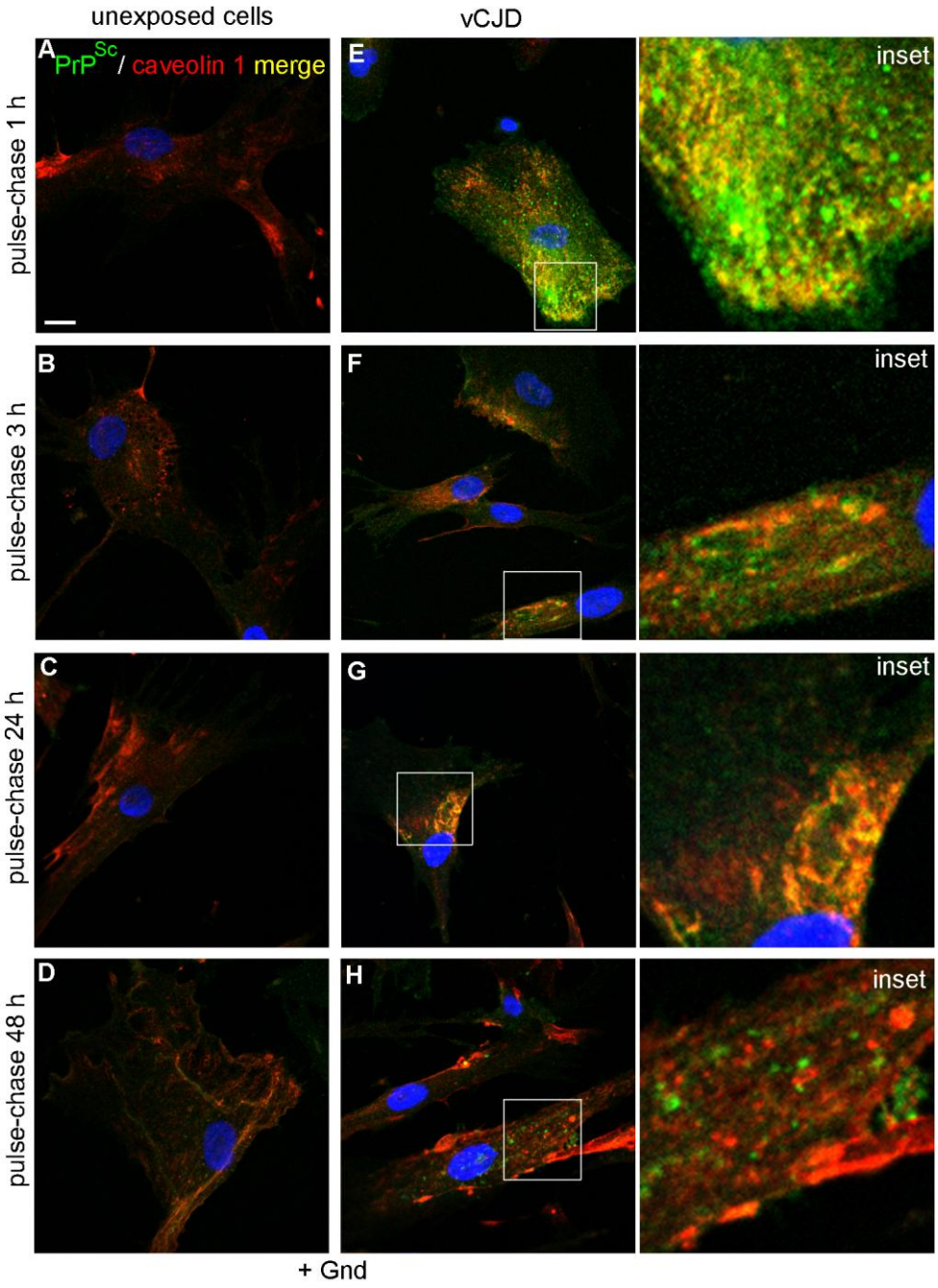


Figure 3.58: PrP^{Sc} colocalisation with caveolae coated vesicles in "pulse and chase" study (II.)

HK cells were incubated and treated as described in Figure caption 3.57 with the additional inclusion of guanidine pre-treatment step during the immunocytochemistry procedure. The cells were double immunolabeled for PrP (green) and caveolae coated vesicles (red) as was described previously in section 2.12.5. The merge of the channels is shown, any colocalisation of PrP^{Sc} with caveolae-coated vesicles is indicated by yellow and the high magnifications of the boxed areas in (E-H) are shown right (inset). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

From this study it is apparent that the caveolae-coated vesicles are involved in the PrP^{Sc} internalisation not only at the early (1 and 3 hours), but also at the later time points of endocytosis (24 hours). However, after 48 hours, the PrP^{Sc} signal could not be considered to be colocalised with caveolae-coated vesicles.

3.7.3.7 PrP^{Sc} colocalisation with clathrin coated pits in "pulse and chase" study (I.)

Having previously observed the possible role of clathrin-coated pits in the endocytosis of the exogenous PrP^{Sc} (section 3.7.3.4), the same "pulse and chase" experimental design as above, but with cells double immunolabeled for PrP (green) and clathrin-coated pits (red) with clathrin antibody was analysed (Figure 3.59). The cells were either unexposed (A-D) or vCJD exposed (E-H, insets of the boxed areas). The merge of the channels is shown. The exogenous PrP^{Sc} was clearly present in cells exposed to vCJD (E-H, bright green). Some positive colocalisation of the exogenous PrP^{Sc} with clathrin-coated pits suggesting involvement in endocytosis in cells exposed to vCJD brain material could be observed at all examined time points (E-H, yellow). However, the involvement appears to be less intense then the endocytosis via caveolae-coated vesicles observed earlier (section 3.7.3.5; Figure 3.57; E-H).

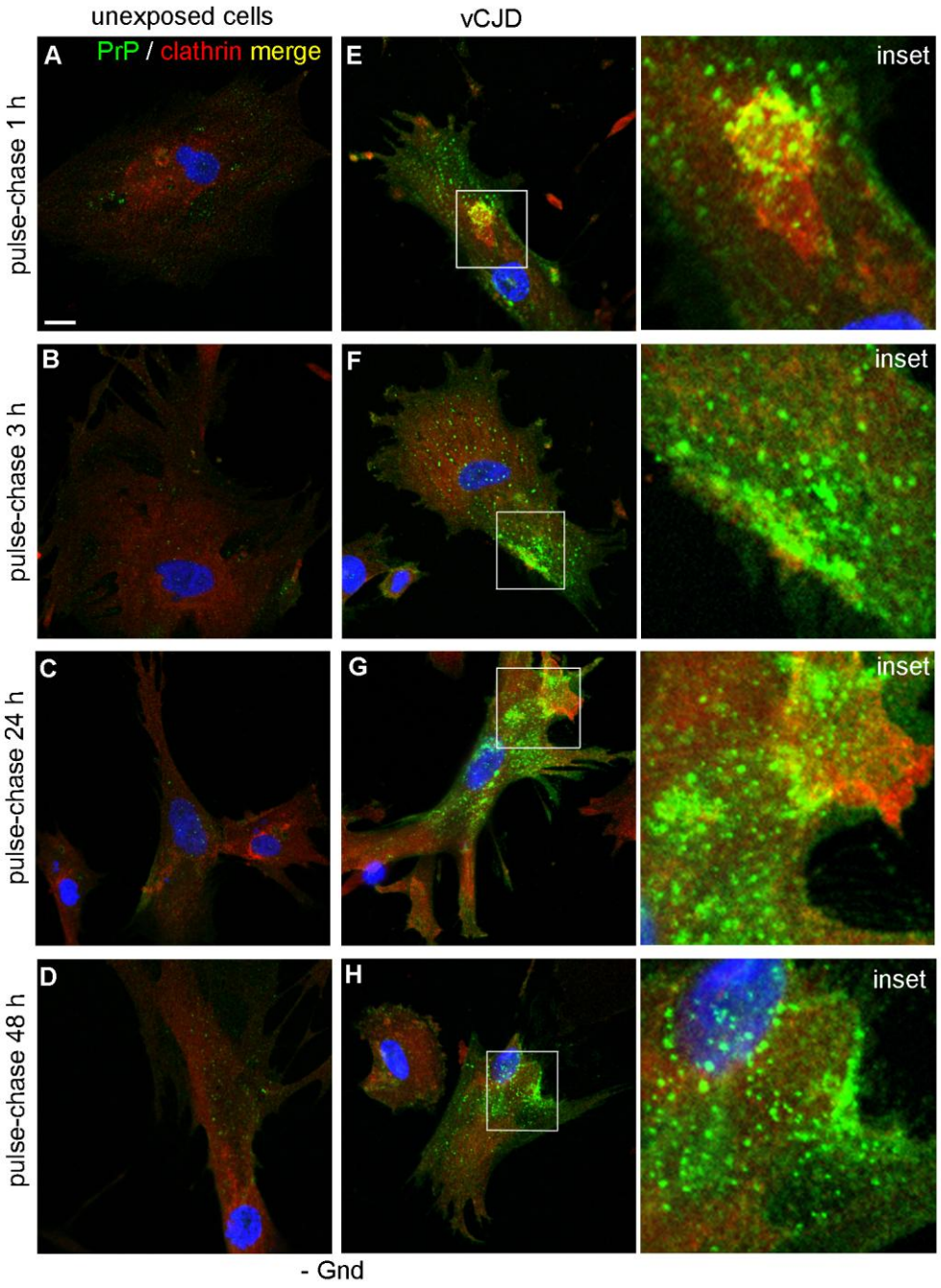


Figure 3.59: PrP^{Sc} colocalisation with clathrin coated pits in "pulse and chase" study (I.)

HK cells were incubated with medium containing 6H4, either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and cells were double immunolabeled for PrP (green) and clathrin coated pits (red) as was described previously in section 2.12.5. The merge of the channels is shown and any colocalisation of PrP with clathrin-coated pits appeared yellow. Insets represent magnifications of the boxed areas in (E-H). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.7.3.8 PrP^{Sc} colocalisation with clathrin coated pits in "pulse and chase" study (II.)

In order to more precisely determine and quantify the involvement of clathrin-coated pits in the uptake of PrP^{Sc}, the guanidine pre-treatment generally used to diminish PrP^{C} and accentuate the PrP^{Sc} signal was used in the immunostaining protocol (Figure 3.60). The same experimental design of the "pulse and chase" assay as in 3.7.3.7 was used. The merge of the channels is shown, PrP is shown (green) and the clathrin-coated pits are shown (red). Any positive colocalisation of PrP^{Sc} and clathrin-coated pits is in yellow and this could be weakly observed at the 24 (G) and 48 hours (H) of "chasing" period in the HK cells exposed to vCJD spiked medium. The result supports the data observed earlier showing the peak of PrP^{Sc} internalisation via clathrin-coated pits at the later times after exposure (24 and 48 hours).

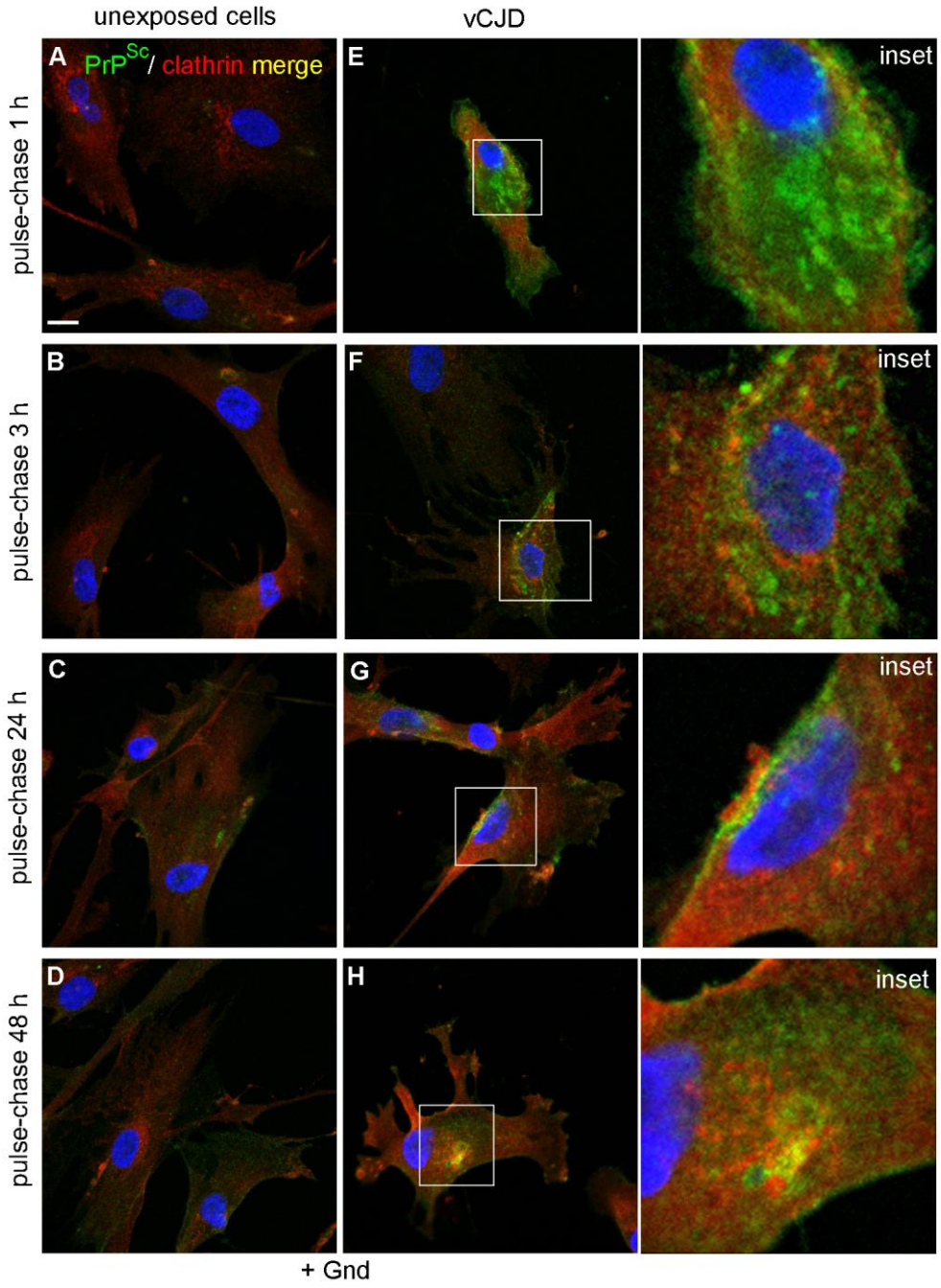


Figure 3.60: PrP^{Sc} colocalisation with clathrin coated pits in "pulse and chase" study (II.)

HK cells were incubated and treated as described in Figure caption 3.59 including a guanidine pre-treatment step during immunocytochemistry procedure. The cells were double immunolabeled for PrP (green) and clathrin coated pits (red) as was described previously in section 2.12.5. The merge of the channels is shown, any colocalisation of PrP^{Sc} and clathrin coated pits appeared yellow and the high magnifications of the boxed areas (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue) and the scale bar, 20 µm.

3.7.4 Summary

- The use of immunocytochemistry to study internalisation of vCJD PrP^{Sc}-6H4 complexes in HK cells has provided the first direct evidence that both caveolae and clathrin-mediated endocytic pathways may be involved in the uptake process of exogenous PrP^{Sc} into the cell.
- The evidence here further suggests that the kinetics of uptake differ, with caveolae-mediated uptake preceeding that of clathrin coated pits.

3.8 TRAFFICKING OF PrP^{Sc} TAKEN UP BY HK CELLS ANALYSED BY IMMUNOCYTOCHEMISTRY

3.8.1 Objectives

To investigate the intracellular fate and the subcellular sites of accumulation of the exogenous prion protein endocytosed by HK cells.

3.8.2 Rationale

The earlier observations (section 3.3) showed that exposure of HK cells to prion infected brain material was not able to establish a long term prion infection in these cells, although they possess all the required components (section 3.2) to support PrP^{Sc} formation. Interestingly, these cells also showed high levels of PrP^{C} (section 3.1.3), and to have the mechanisms to allow a rapid uptake of the infectious brain material (section 3.4, 3.5 and 3.7). However, the findings of the recovery studies (section 3.6) that HK cells are also able to clear the endocytosed PrP^{Sc}, suggests that they have mechanism to degrade, or otherwise process the infectious material. Interestingly, the endocytosed PrP^{Sc} material could not be detected to be shed back into the culture media (section 3.6). An earlier study (section 3.5) showed that exogenous PrP^{Sc} taken up by HK cells was mostly found in the juxtanuclear region of cytoplasm. Therefore sub-cellular localisation of exogenous PrP^{Sc} and identification of the cell organelles involved in HK cells was attempted by ICC.

3.8.3 Experimental results

Identifying the intracellular compartments and the mechanisms involved in the trafficking of the prion material is important for characterisation of the exact intracellular localisation of the exogenous PrP^{Sc} after being taken up.

Therefore, to investigate the subcellular distribution of the exogenous PrP^{Sc} in regards to its cell organelle colocalisation in prion exposed HK cells, a study defining the exogenous prion protein trafficking through HK cells was performed and examined by immunofluorescence microscopy. Standard conditions of fixation and permeabilisation, with a short pre-treatment with guanidine thiocyanate (Gnd), to expose the PrP^{Sc}-specific epitopes and diminish PrP^{C}, were used. Moreover, this study also employed the previously used fluorescent signal thresholding procedure to reveal the intracellular distribution of all PrP^{Sc} taken up by cells exposed to brain spiked medium. Detection of prion protein was performed using the anti-prion protein primary antibody 8H4 or 6H4. Antibodies were carefully selected for the detection of PrP and cell organelles in the double immunolabeling method based on their properties and their use in published reports.

The ICC colocalisation data shown in this chapter are representative of seven independent non-identical experiments. The differences included the time points assayed and the origin of the inoculum used.

3.8.3.1. Cell organelle structures of HK cells examined by immunofluorescence

The possibility of exogenous PrP^{Sc} trafficking via early endosomes, as tested using the EEA1 antibody (Figure 3.61; A) (Wilson *et al.*, 2000; Peters *et al.*, 2003; Campana *et al.*, 2005; Pimpinelli *et al.*, 2005; Marijanovic *et al.*, 2009; Veith *et al.*, 2009) was investigated as first.

Based on findings from earlier observations (section 3.5.3.9-10), exogenous PrP^{Sc} taken up by HK cells is mostly found in the perinuclear region that partially overlaps the Golgi complex or endoplasmic reticulum. Therefore, it was of interest to evaluate their co-localisation by using giantin - a Golgi complex specific monoclonal antibody (Figure 3.61; B) (Campana *et al.*, 2005; Dron *et al.*, 2009; Marijanovic *et al.*, 2009) and calnexin - an endoplasmic reticulum specific monoclonal antibody (Figure 3.61; C) (Campana *et al.*, 2005). Note that the calnexin antibody gave only a very faint signal, even when the antibody concentration was increased up to four times that recommendation by the manufacturer.

The rab11A antibody, labeling recycling endosomes (Figure 3.61; D) (Urbe *et al.*, 1993; Ullrich *et al.*, 1996; Marijanovic *et al.*, 2009) localised to juxtanuclear and peripheral endocytic vesicles and regulating vesicular transport, was also used for examining colocalisation with the exogenous PrP^{Sc}.

To further clarify PrP^{Sc} clearance (section 3.6) from HK cells and taking into account the previous observation that lysosomal proteases would, to some extent, degrade PrP^{Sc} in prion infected cells in culture (Supattapone *et al.*, 2001), or in cell culture of bovine macrophages exposed to mouse adapted scrapie and BSE (Sassa *et al.*, 2010), it was of interest to examine whether the PrP^{Sc} clearance could be ascribed to lysosomal degradation process in HK cells. The antibodies LAMP1 (Figure 3.61; E) (Sun *et al.*, 1997; Brauer *et al.*, 2004; Wilson *et al.*, 2004; Falcon-Perez *et al.*, 2005; Kristiansen *et al.*, 2007; Godsave *et al.*, 2008; Okemoto-Nakamura *et al.*, 2008) and LAMP2b (Figure 3.61; F) were used to label lysosomes in this experiment.

An Alexa 546-conjugated secondary antibody was used throughout.

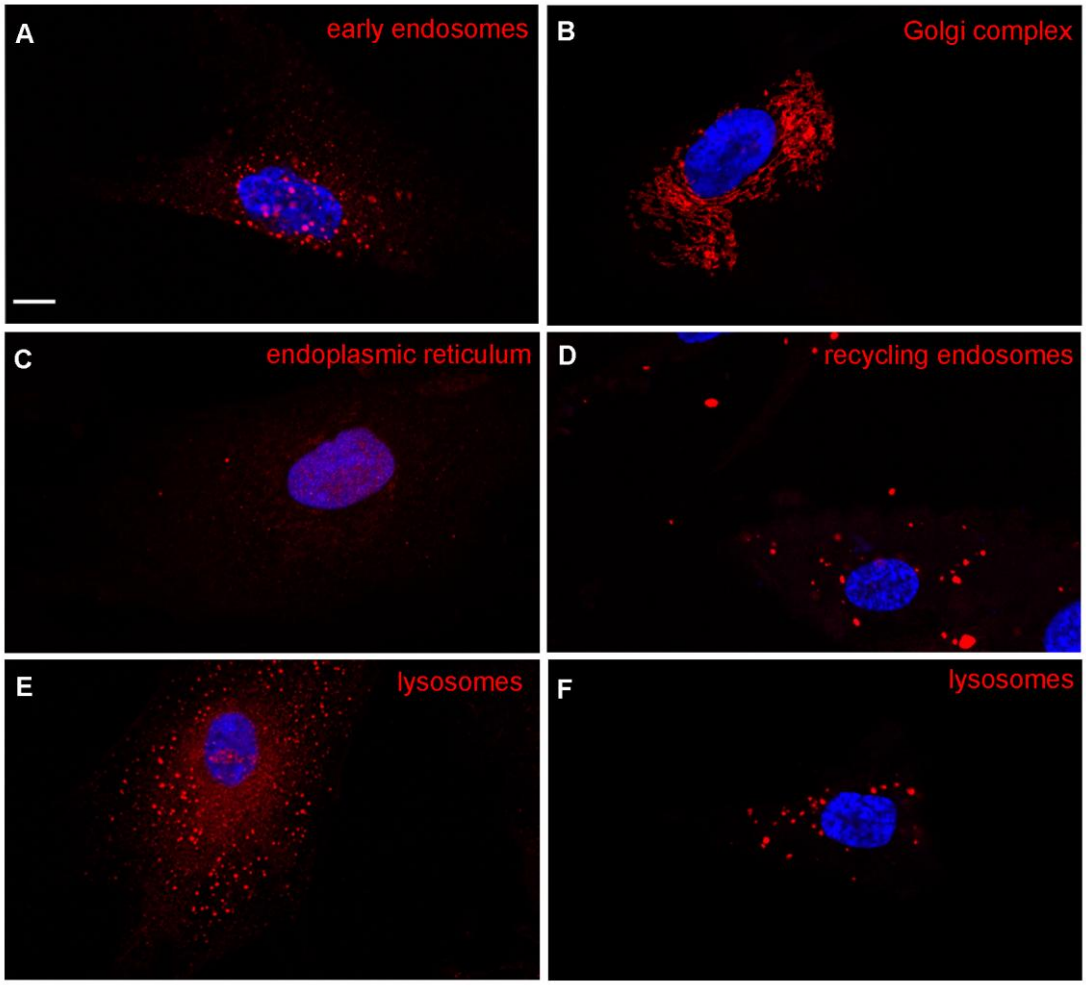


Figure 3.61: Immunostaining of organelles chosen for the trafficking study of exogenous PrP^{Sc} in HK cells
HK cells were washed, fixed, permeabilised and immunolabeled (following the standardised ICC protocol as described in section 2.12.2) for early endosomes (A) with EEA1 antibody, the Golgi complex (B) with giantin, endoplasmic reticulum (C) with calnexin, recycling endosomes (D) with rab11A antibody, lysosomes with LAMP1 antibody (E) and LAMP2b antibody (F). The cells were then incubated with Alexa 546 (red) and the nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.8.3.2 PK pre-treatment has a destructive effect on the cell organelle integrity

In this study, the PK pre-treatment during the immunocytochemical procedure had to be omitted as it turned out to severely affect the structurality of cell organelles. The characteristic structures (without PK pre-treatment) of the Golgi complex (Figure 3.62; A) and the endoplasmic reticulum (Figure 3.62; C) in HK cells are shown. The

PK pre-treatment caused the cell organelle structures to collapse (Figure 3.62; B) and the relevance of observations upon PK pre-treatment therefore could be not evaluated accurately (Figure 3.62; D).

Fortunately, PK pre-treatment was not essential, as PrP^{Sc} could be easily distinguished from PrP^{C} when necessary by a guanidine pre-treatment and using uniform signal threshold in the course of all confocal microscope analysis, to maximise the PrP^{Sc} detection. It is important to note that the Gnd pre-treatment neither affected the cell organelle structures, nor interfered with the PrP^{C} staining, whereas it was necessary for the detection and enhancement of the PrP^{Sc} signal. All further experiments in this section were undertaken without PK pre-treatment.

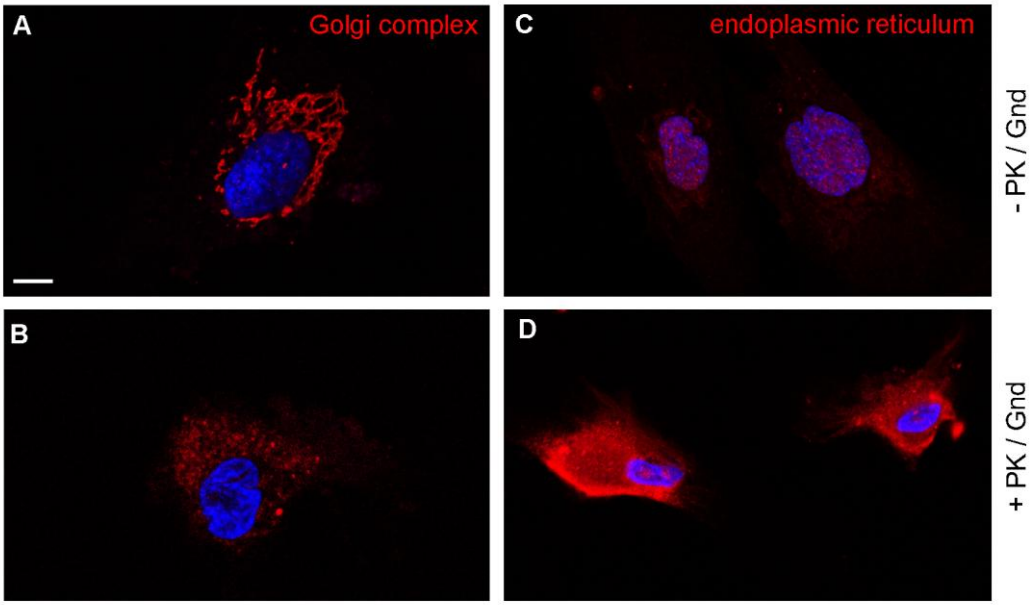


Figure 3.62: Proteinase K pre-treatment has a severe destructive effect on the cell organelles integrity
The HK cells were immunostained as described previously (section 2.12.3) and either allowed to remain not pre-treated with PK/Gnd (A, C) or treated (B, D). Antibody giantin was used to visualise the Golgi complex (A, B) and calnexin to visualise the endoplasmic reticulum (C, D) (red). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

To investigate the intracellular fate of exogenous PrP^{Sc} in HK cells exposed to prion diseased brain homogenates, a series of uptake experiments were performed. It is important to note that pre-treatment of the examined cells with guanidine was used in these studies only when specifically mentioned.

3.8.3.3 Investigation of PrP^{Sc} colocalisation with early endosomes after continuous exposure of HK cells to vCJD brain material

The first investigation was focused on whether endocytosed exogenous PrP^{Sc} intersects with the classical early endocytic recycling pathway involving early endosomes, the Golgi complex or the endoplasmic reticulum. This was examined at four time points from the 1-72 hours of continuous exposure to prion diseased brain spiked medium.

The HK cells were subjected to continuous exposure to vCJD brain spiked medium for 1, 24, 48 and 72 hours and labelled with 8H4 (Figure 3.63; left column) and 6H4 (Figure 3.63; right column) anti-prion protein primary antibody.

The cell-associated PrP signal (green) could be observed, as in earlier studies, to increase in the cells with the time of exposure. Identical results for cell-associated PrP, (regarding time-dependent uptake of PrP^{Sc} from brain spiked media), were observed by both anti-PrP antibodies. Early endosomes are visualised in red colour. All micrographs represent the merge of the green and red channels and any positive colocalisation of early endosomes (red) with PrP (green) should appear as yellow. No obvious evidence of positive colocalisation of PrP^{Sc} with early endosomes was seen at any time point of continuous exposure to vCJD spiked medium, although some limited evidence of overlap at the 24 hours time point analysed by 6H4 antibody was detected (Figure 3.63; right column).

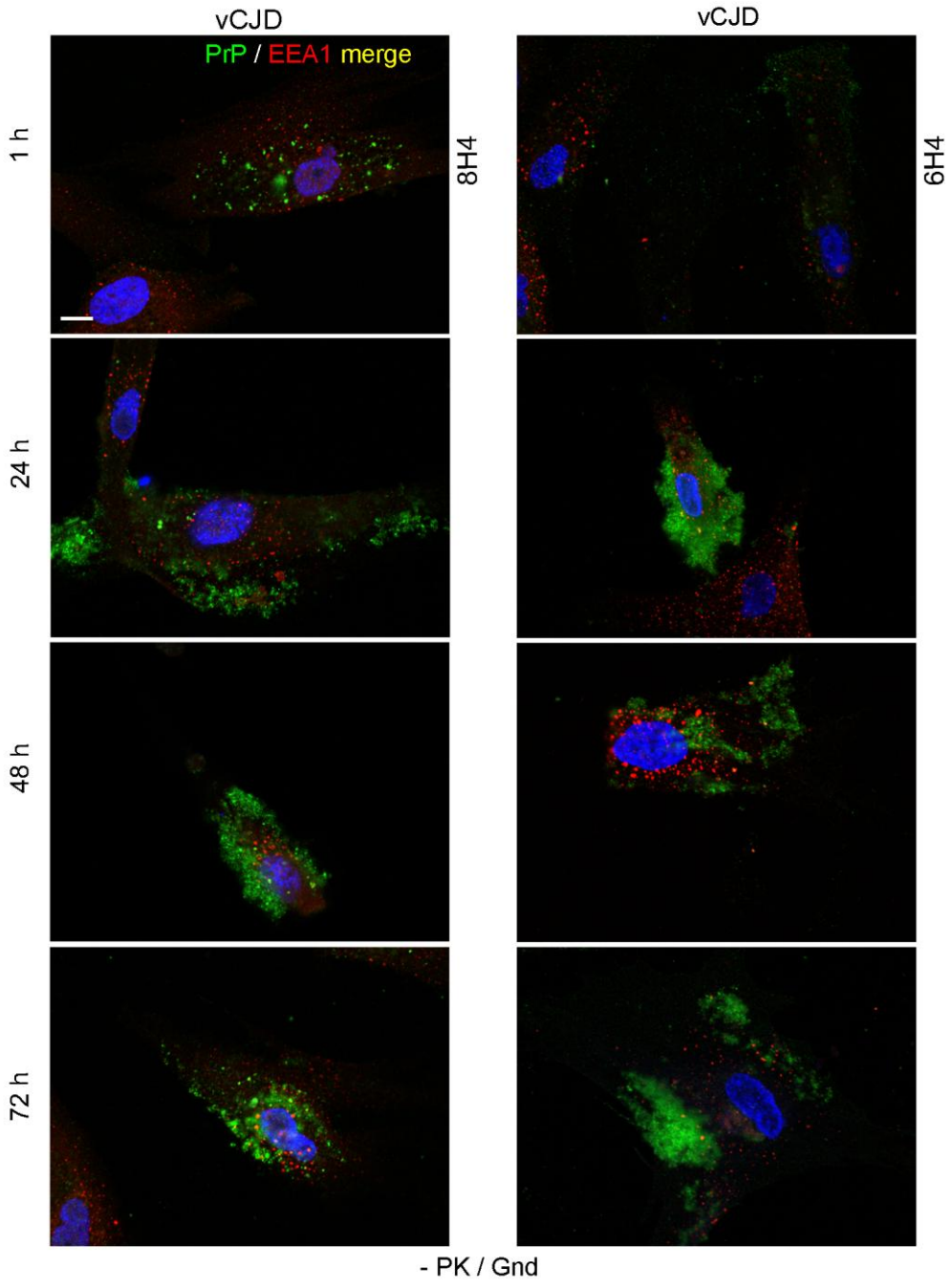


Figure 3.63: Investigation of PrP^{Sc} colocalisation with early endosomes after continuous exposure to vCJD brain material

HK cells were continuously exposed to 1% vCJD brain spiked medium for 1, 24, 48 and 72 hours. The cells were then washed and double immunolabeled for PrP with antibody 8H4 (left column) or 6H4 (right column) (green) and early endosomes antibody EEA1 (red). The merge of the channels is shown. The nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

3.8.3.4 Investigation of PrP^{Sc} colocalisation with the Golgi complex after continuous exposure of HK cells to vCJD brain material

The same experimental design was used to examine whether the endocytosed exogenous PrP^{Sc} is trafficked to the Golgi complex during the four examined time points (1-72 hours) of continuous exposure to prion infected brain spiked medium (Figure 3.64). The anti-PrP primary antibodies 8H4 (Figure 3.64; left column) and 6H4 (Figure 3.64; right column) were used to detect PrP (green). The Golgi complex was visualised with antibody giantin (red). All micrographs represent the merge of the green and red channels and any positive colocalisation of the Golgi complex (red) with PrP (green) should appear as yellow.

The time dependent increase of cell-associated exogenous PrP^{Sc} was unambiguous and uniform when visualised by both anti-PrP protein antibodies. No obvious evidence of colocalisation of the exogenous PrP^{Sc} with the Golgi complex could be observed at any examined time point using either 8H4 or 6H4 antibody.

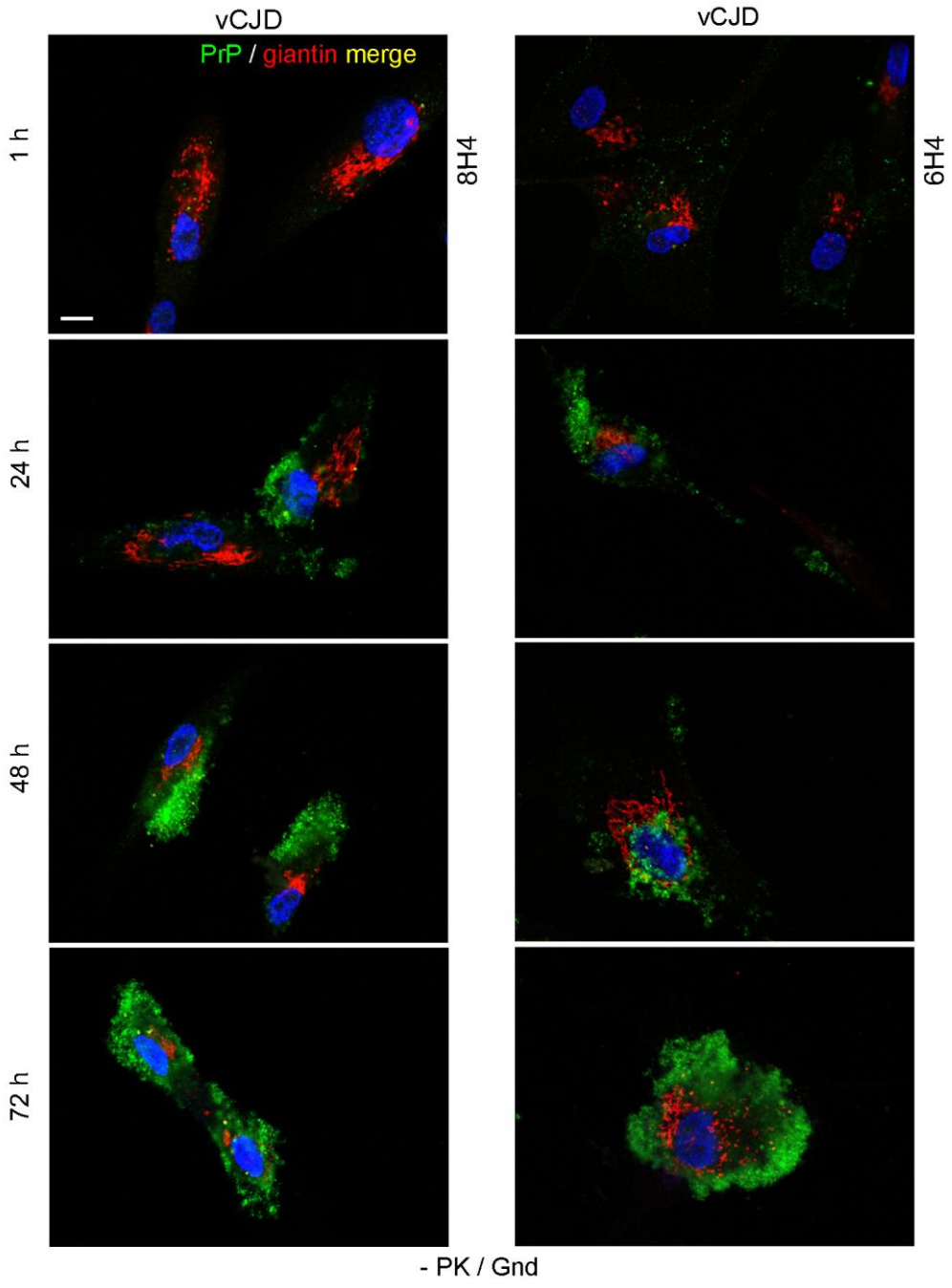


Figure 3.64: Investigation of PrP^{Sc} colocalisation with the Golgi complex after continuous exposure of HK cells to vCJD brain material
HK cells were continuously exposed to 1% vCJD brain spiked medium for 1, 24, 48 and 72 hours. The cells were then washed and double immunolabeled for PrP with antibody 8H4 (left column) or 6H4 (right column) (green) and the Golgi complex with antibody giantin (red). The merge of the channels is shown. The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.5 Investigation of PrP^{Sc} colocalisation with the endoplasmic reticulum after continuous exposure of HK cells to vCJD brain material

Next the question of whether the endoplasmic reticulum contains any endocytosed exogenous PrP^{Sc} was addressed (Figure 3.65). The experimental design was the same as applied previously. PrP was detected using 8H4 (Figure 3.65; left column) and 6H4 antibody (Figure 3.65; right column) (green). The endoplasmic reticulum was visualised using antibody calnexin (faint red). The merge of the channels is shown. The time dependent increase of cell-associated PrP^{Sc} after continuous exposure to vCJD brain spiked medium was clearly shown (green). The exogenous PrP^{Sc} did not obviously colocalise with endoplasmic reticulum in HK cells at any examined time point, although calnexin staining was faint.

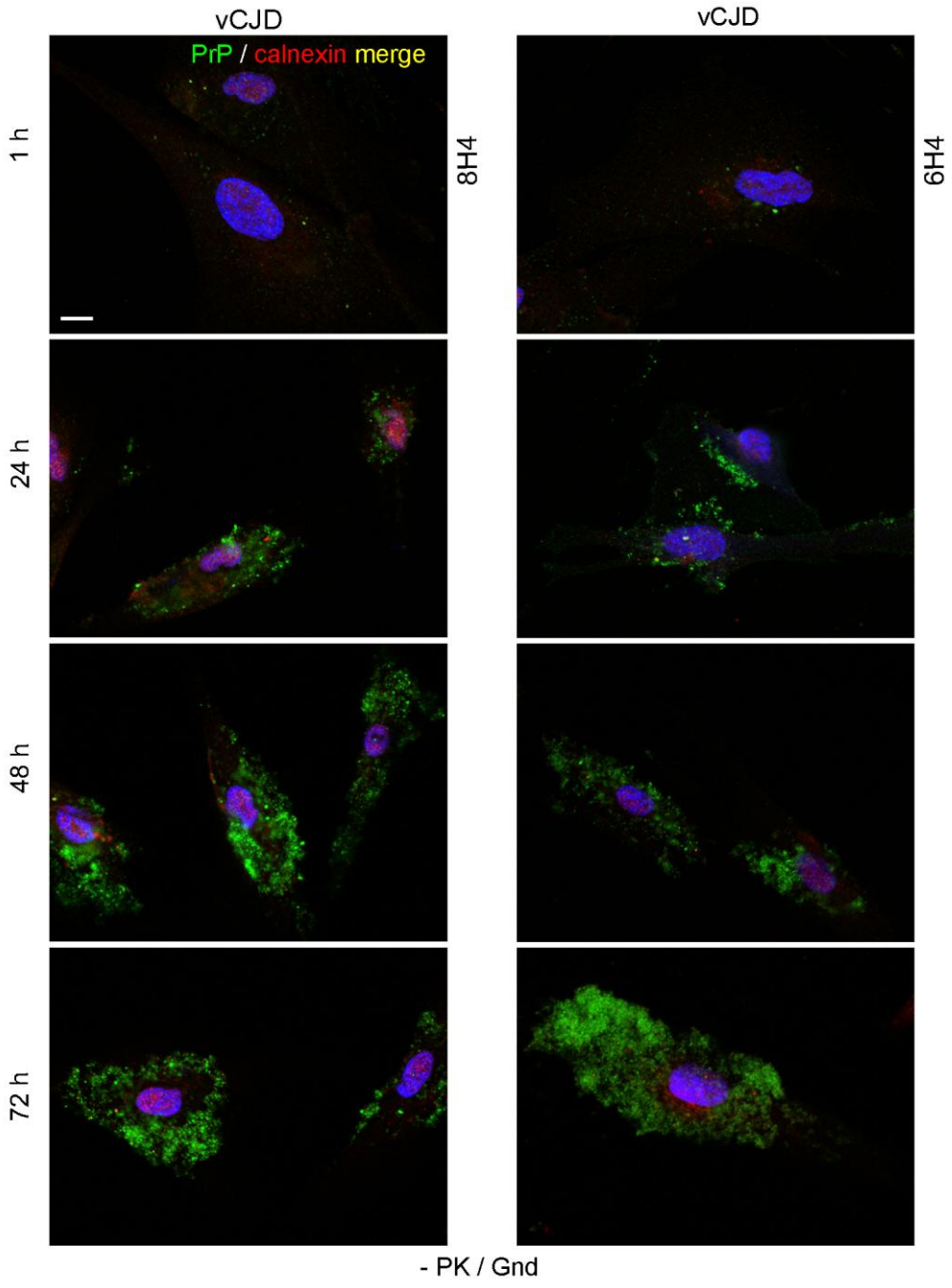


Figure 3.65: Investigation of PrP^{Sc} colocalisation with the endoplasmic reticulum after continuous exposure of HK cells to vCJD brain material
HK cells were continuously exposed to 1% vCJD brain spiked medium for 1, 24, 48 and 72 hours. The cells were then washed and double immunolabeled for PrP with antibody 8H4 (left column) or 6H4 (right column) (green) and the endoplasmic reticulum with antibody calnexin (red). The merge of the channels is shown. The nuclei were counterstained with DAPI (blue). The scale bar represents 20 µm.

In the above investigations, no evidence of exogenous PrP^{Sc} colocalisation with markers of the endocytic pathway, such as EEA1, giantin and calnexin was found. To demonstrate that these findings were not the result of a technical artefact, it was decided to perform a series of "pulse-chase" studies (described in sections 2.11.2.3 and in section 2.12.5). Therefore, based on the assumption that the trafficking of endocytosed prion material could be more precisely evaluated by the "pulse-chase" study, it was proposed to repeat the investigation of the PrP^{Sc} colocalisation with the early endosomes, the Golgi complex and the endoplasmic reticulum by applying this principle (Figure 3.66-3.68). Firstly, HK cells were "pulsed" with complete (unspiked) medium containing 6H4 primary antibody (1 µg/ml) – as a control (Figure 3.66-3.68; A-D) or with vCJD PrP^{Sc}-6H4 complexes (Figure 3.66-3.68; E-H, insets of boxed areas). The "pulse" period was composed of 30 minutes incubation at 4 °C and additional 15 minutes incubation at 37 °C to initiate the internalisation of the antibody-antigen complex. The cells were then extensively washed and incubated with a fresh complete medium (not containing 6H4) for the desired "chase" time period before double labelling immunocytochemistry for PrP and cell organelles was carried out (as described in section 2.12.5).

3.8.3.6 Investigation of PrP^{Sc} colocalisation with early endosomes in the "pulse and chase" study

The cells were double immunolabeled for PrP (green) and early endosomes (red) with the EEA1 antibody (Figure 3.66) at 1 (A, E); 3 (B, F); 24 (C, G) and 48 hours (D, H) of the "chasing" period. The merge of the channels is shown and any positive colocalisation of endocytosed exogenous PrP^{Sc} with early endosomes should appear as yellow.

An overlap or positive colocalisation of exogenous PrP^{Sc} with early endosomes could be observed in the form of few yellow dots.

The difference in the PrP signal (green) of the unexposed cells (dull green) (A-D) compared to the PrP signal of the cells exposed to prion diseased medium (bright green) (E-H) indicates that the vCJD PrP^{Sc}-6H4 is taken up together as a complex, rather than the 6H4 primary antibody being taken up on its own.

At steady-state (data not shown) and also after "pulse-chase" when HK cells were "pulsed" with complete (unspiked) medium containing 6H4 mAb (Figure 3.66; A-D), the early endosome antibody EEA1 did not colocalise with endogenous cellular PrP^{C} (even when the fluorescence signal threshold was set to extreme values). However, it is possible that level of PrP^{C} in the early endosomes was below the detection limit. In this respect, the investigations obtained thus far showed no evidence of endogenous PrP^{C} and some (but inconclusive) evidence that the endocytosed exogenous PrP^{Sc} may be trafficked via early endosomes in HK cells. Whether this colocalisation was real or random was assessed by a quantitative image analysis study in this thesis (section 2.14 and 3.8.3.19).

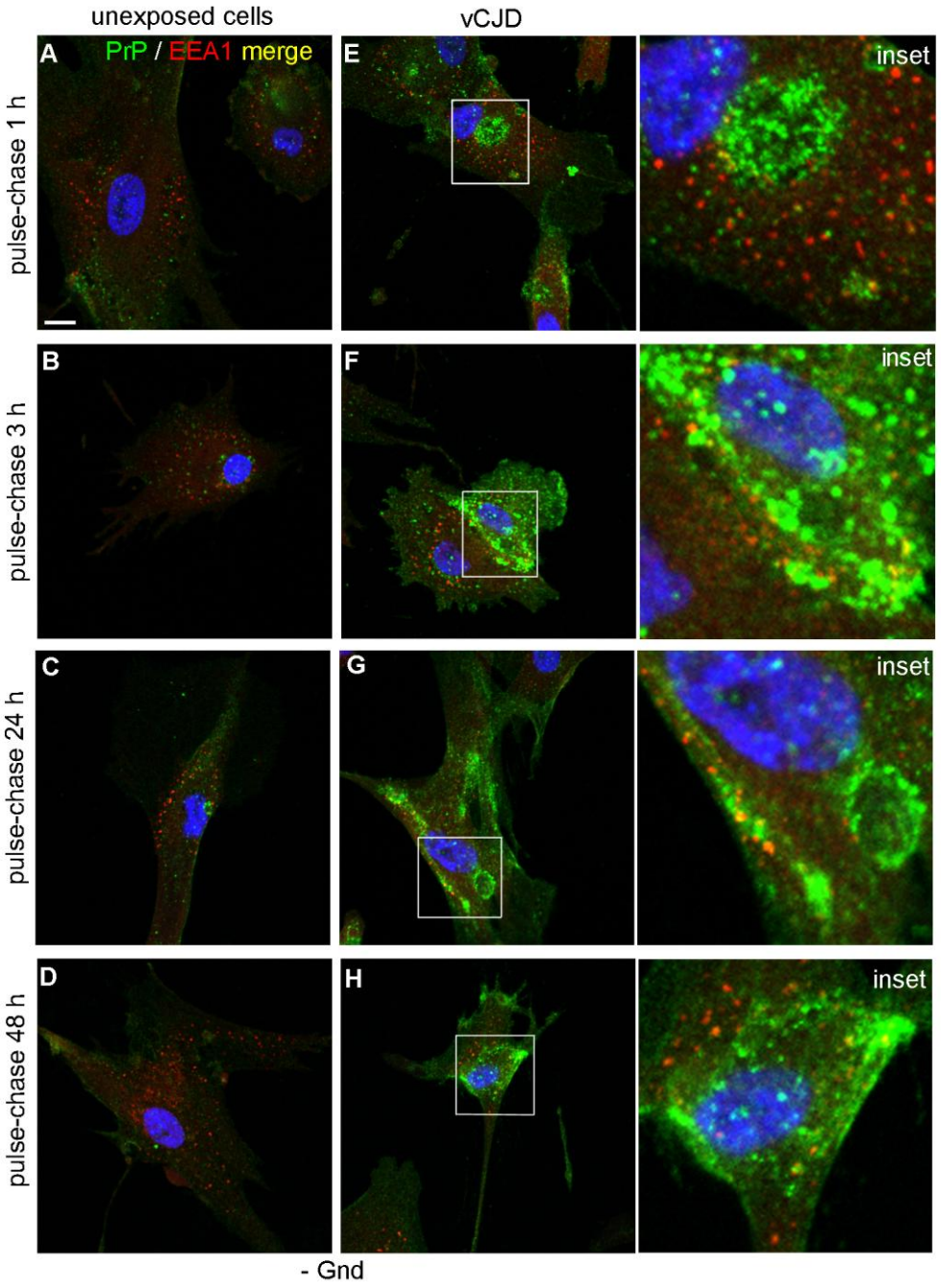


Figure 3.66: Investigation of PrP^{Sc} colocalisation with early endosomes in the "pulse and chase" study

HK cells were incubated with medium containing 6H4 (1 µg/ml), either control (A-D) or spiked with 1% vCJD brain (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and the cells were double immunolabeled for PrP (green) and early endosomes (red) as was described previously in section 2.12.5. The merge of the channels is shown, any colocalisation appeared in yellow colour and the high magnification of the boxed areas in (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.7 Investigation of PrP^{Sc} colocalisation with the Golgi complex in the "pulse and chase" study

The next "pulse-chase" study evaluated colocalisation of exogenous PrP^{Sc} with the Golgi complex (Figure 3.67). The experimental design was the same as previously applied (section 3.8.3.6), except the cell organelle examined was the Golgi complex labelled with antibody giantin (red) at 1 (A, E); 3 (B, F); 24 (C, G) and 48 hours (D, H) of the "chasing" time period. Merge of the channels is shown. The exogenous PrP^{Sc} (green) in cells spiked with vCJD brain homogenate was clearly present intracellularly at all examined time points and increasing in a time dependent manner (E-H, inset – high magnification of the boxed areas).

However, no evidence of positive colocalisation (yellow) of endocytosed exogenous PrP^{Sc} with the Golgi complex was observed in cells exposed to vCJD medium (Figure 3.67; E-H). This observation is consistent with the earlier results obtained after continuous exposure to brain spiked medium (section 3.8.3.4). Moreover, no positive colocalisation of the HK cell's own PrP^{C} with the Golgi complex could be observed in the conditions used (Figure 3.67; A-D).

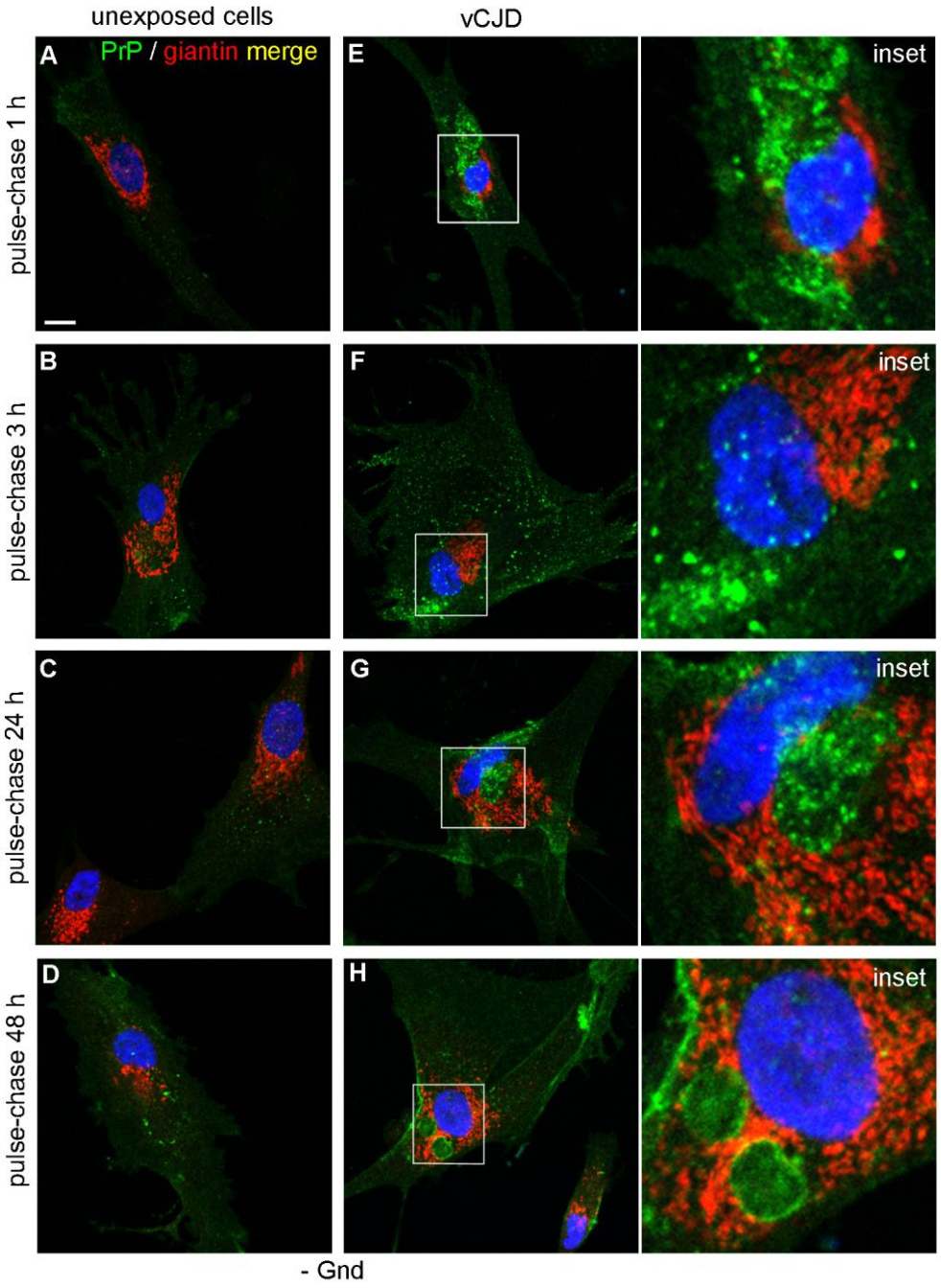


Figure 3.67: Investigation of PrP^{Sc} colocalisation with Golgi complex in the "pulse and chase" study

HK cells were incubated with control medium containing 6H4 (A-D) or 1% vCJD-6H4 (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and cells were double immunolabeled for PrP (green) and the Golgi complex (red) as was described previously in section 2.12.5. The merge of the channels is shown, any colocalisation appeared as yellow and the high magnification for (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.8.3.8 Investigation of PrP^{Sc} colocalisation with the endoplasmic reticulum in the "pulse and chase" study

The "pulse-chase" study also re-examined the possible colocalisation of endocytosed exogenous PrP^{Sc} with endoplasmic reticulum in the HK cells (Figure 3.68). Experimental design of this "pulse-chase" assay was used as before (sections 3.8.3.6-7). The endoplasmic reticulum was detected with antibody calnexin (red). The merge of the channels is shown. The exogenous PrP^{Sc} in cells spiked with vCJD brain homogenate was present at all examined time points (E-H, inset – high magnification of the boxed areas) (green). No convincing evidence of positive colocalisation (yellow) of endocytosed exogenous PrP^{Sc} within the endoplasmic reticulum of HK cells exposed to vCJD medium could be observed (Figure 3.68; E-H). This observation is consistent with our earlier results (section 3.8.3.5) although immunostaining of the endoplasmic reticulum with the calnexin antibody was faint.

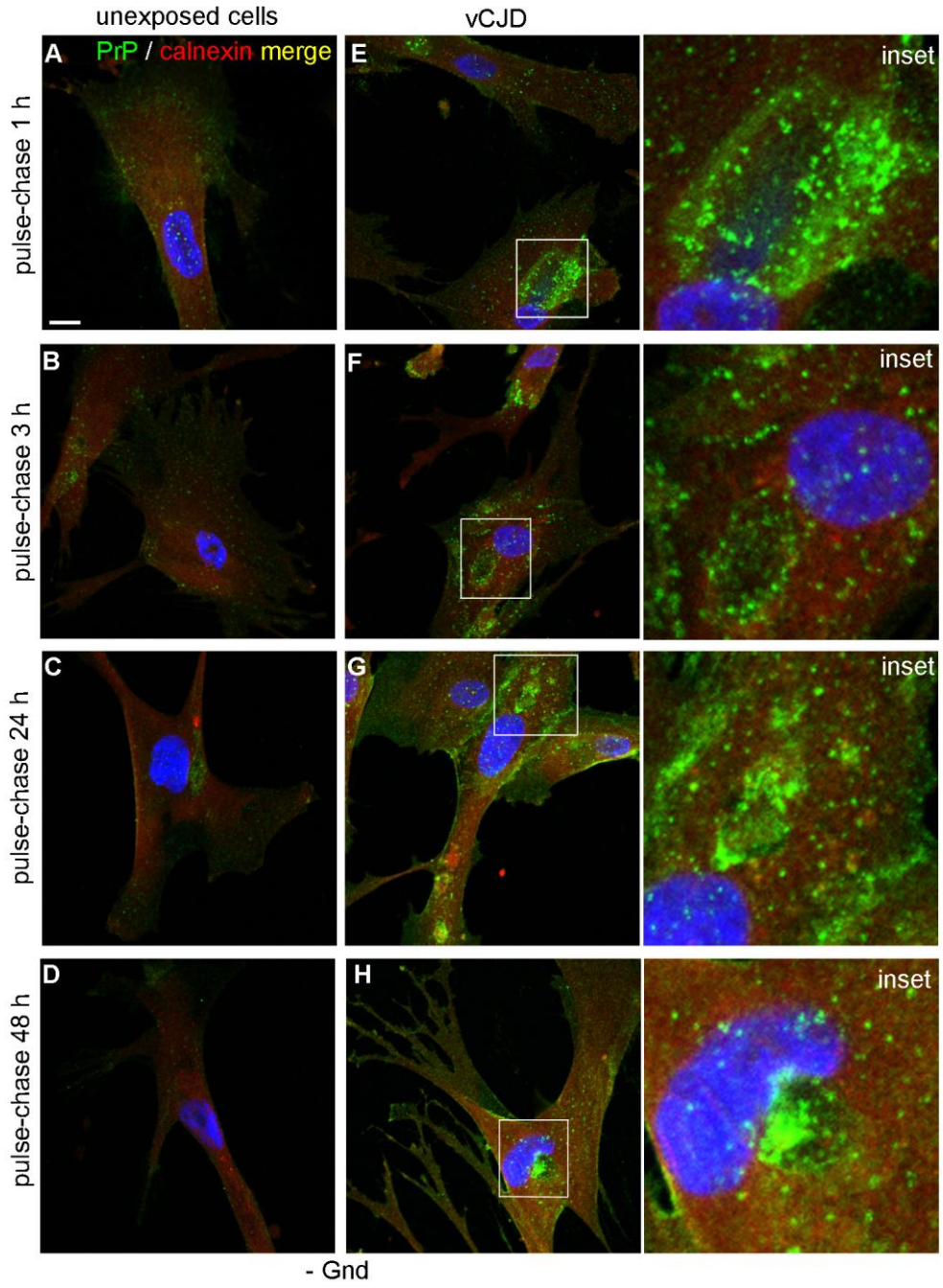


Figure 3.68 Investigation of PrP^{Sc} colocalisation with the endoplasmic reticulum in the "pulse and chase" study

HK cells were incubated with medium containing 6H4, either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C - pulse. Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – chase. The medium was then aspirated and the cells were double immunolabeled for PrP (green) and the endoplasmic reticulum (red) as was described previously in 2.12.5. The merge of the channels is shown, any colocalisation appeared yellow and the high magnification of the boxed areas in (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

The immunocytochemical studies of exogenous PrP^{Sc} trafficking and colocalisation with early endosomes, the Golgi complex and the endoplasmic reticulum were carried out either after continuous exposure to brain spiked medium or by the "pulse-chase" format. Confocal analysis showed that, in juxtanuclear areas, Golgi complex or endoplasmic reticulum and PrP^{Sc} labelling occured in the same general region, but did not truly colocalise. It was concluded that these compartments did not contain substantial amounts of endocytosed PrP^{Sc}.

It was therefore thought that exogenous PrP^{Sc} might be trafficked by the late endosomal/lysosomal route after being endocytosed by caveolae-coated vesicles.

3.8.3.9 Investigation of PrP^{Sc} colocalisation with lysosomes after continuous exposure of HK cells to vCJD brain material (I.)

HK cells were exposed to vCJD brain spiked medium for 1-72 hours and then double immunolabeled with anti-PrP (green) primary antibody 8H4 (Figure 3.69; left column) and 6H4 (Figure 3.69; right column) and the anti-lysosomal antibody LAMP2b (red). The merge of the channels is shown and positive colocalisation (yellow) of endocytosed exogenous PrP could be clearly observed when the 8H4 primary antibody was used (left column). In this case, when the 6H4 antibody was used (right column) the positive colocalisation could be also observed, but only after the fluorescence signal threshold was set to a higher level than for 8H4. The same signal threshold are shown for both antibodies to maintain consistency within the studies.

It is important to note that the HK cells challenged with vCJD brain material showed intensely positive colocalisation of endocytosed exogenous PrP^{Sc} with lysosomes. It is also interesting to note that number of lysosomal structures within the cells

appeared to increase with the exposure time. Longer exposure times (24-72 hours) gave the most convincing colocalisation.

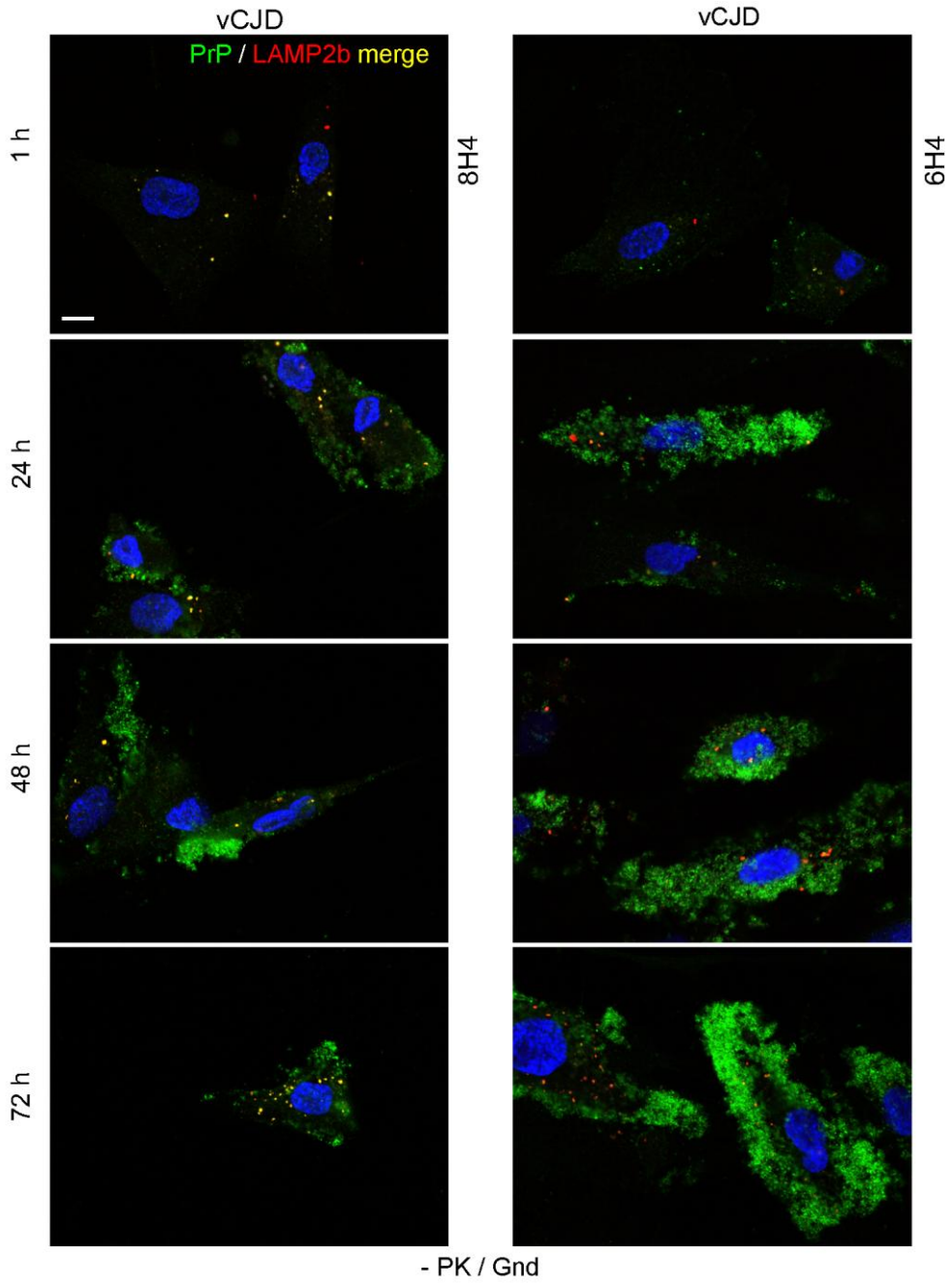


Figure 3.69: Investigation of PrP^{Sc} colocalisation with lysosomes after continuous exposure to vCJD (I.)
HK cells were continuously exposed to 1% vCJD brain spiked medium for 1, 24, 48 and 72 hours. The cells were then washed and double immunolabeled for PrP with antibody 8H4 (left column) or 6H4 (right column) (green) and lysosomes using antibody LAMP2b (red). The merge if the channels is shown, any colocalisation of PrP^{Sc} with lysosomes appeared in yellow colour. The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.10 Investigation of PrP^{Sc} colocalisation within lysosomes after continuous exposure of HK cells to vCJD brain material (II.)

Further study of PrP^{Sc} colocalisation within lysosomes after (1-72 hours) continuous exposure, in a more detailed display (the individual immunostaining channels, as well as the merge channel and inset of the boxed areas of the merge figure) is shown in Figure 3.70. Under standard conditions, the vCJD exposed HK cells were fixed, permeabilised and double immunolabeled for PrP with 8H4 primary antibody (green) (far left) and lysosomes with LAMP2b antibody (red) (left from the centre). Apparent colocalisation of PrP^{Sc} within the lysosomal structures (yellow) can be seen in the merge channels (right from the centre) and higher magnification view is shown in the inset (far right).

The time dependent increase of cell-associated PrP^{Sc} (green) could be clearly observed (column far left). Almost all visible structures labelled as lysosomes were positive for exogenous PrP^{Sc} at 48 and 72 hours post exposure (right from the centre; 48 and 72 hour time point). Again, an exponentially increasing number of cellular lysosomal structures with exposure time (left from the centre, red channel) was observed.

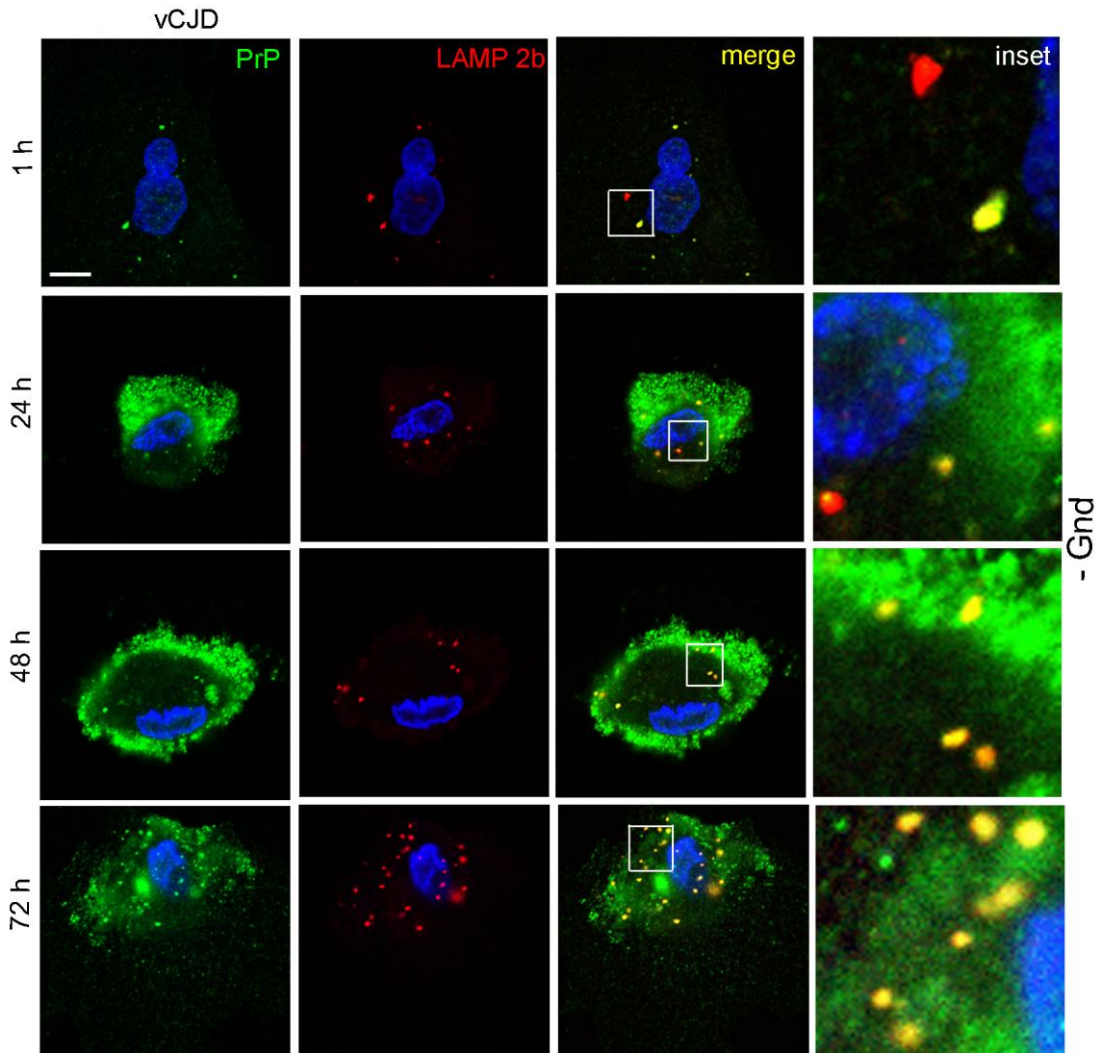


Figure 3.70: Investigation of PrP^{Sc} colocalisation within lysosomes after continuous exposure of HK cells to vCJD brain material (II.)
More detailed display of PrP^{Sc} colocalisation within lysosomes after continuous exposure of HK cells to vCJD brain spiked medium. The cells were incubated and treated same as described previously (Figure descriptions 3.63-3.65), PrP antibody used was 8H4 (green). Individual channels (green/PrP and red/lysosomes) are shown. Yellow colour in merged channels (green and red) indicates colocalisation of PrP^{Sc} with lysosomes. Insets (far right) represent magnification of the boxed areas. Scale bar, 20 µm.

3.8.3.11 Investigation of PrP^{Sc} colocalisation within lysosomes after continuous exposure of HK cells to iCJD, vCJD and AD brain spiked medium

To investigate whether colocalisation of PrP^{Sc} with HK cell lysosomal structures was specific to vCJD, cells exposed to iCJD brain spiked medium (Figure 3.71; Ab and Ac) unexposed control cells (Figure 3.71; Aa) were examined. Cells were

continuously exposed for 48 hours and immunolabeled either under standard conditions (Aa, Ab) or with guanidine pre-treatment (Ac). Strong PrP^{Sc} immunostaining was seen in the lysosomes of iCJD exposed cells. The results were similar to those observed with vCJD and the positive colocalisation of endocytosed exogenous iCJD PrP^{Sc} with cell lysosomes suggests that this event is not specific for vCJD. The PrP^{Sc} colocalisation with lysosomes was more intense in the cells pre-treated with guanidine (Figure 3.71; Ac), indicating the efficiency of the pre-treatment in regards of exposing the PrP^{Sc}-specific epitopes to anti-PrP primary antibody. It was observed that there were fewer numbers of lysosomes in unexposed cells (Figure 3.71; Aa) than in those HK cells exposed to vCJD and iCJD. Furthermore, they were PrP negative using the thresholding criteria.

To determine whether the observed difference in the number of lysosomal structures between brain homogenate exposed and unexposed cells resulted from exposure to brain material, or to exposure to vCJD derived brain material, the HK cells were exposed to AD (Figure 3.71; Ba) and vCJD (Figure 3.71; Bb) brain homogenates for 48 hours. Cells were immunostained under standard conditions with the 6H4 antibody (green) and lysosomal antibody LAMP2b (red). The merge of the channels is shown. The results indicate that the higher numbers of cell lysosomes resulted from cell exposure to brain homogenates rather than CJD brain homogenates. For comparison, see unexposed cells (Figure 3.71; Aa) and cells exposed to AD brain spiked medium (Figure 3.71; Ba). This investigation suggested that the observed higher number of cell lysosomes is caused by the cell exposure to any brain material, rather than being specifically related to exposure to prion diseased brain homogenate.

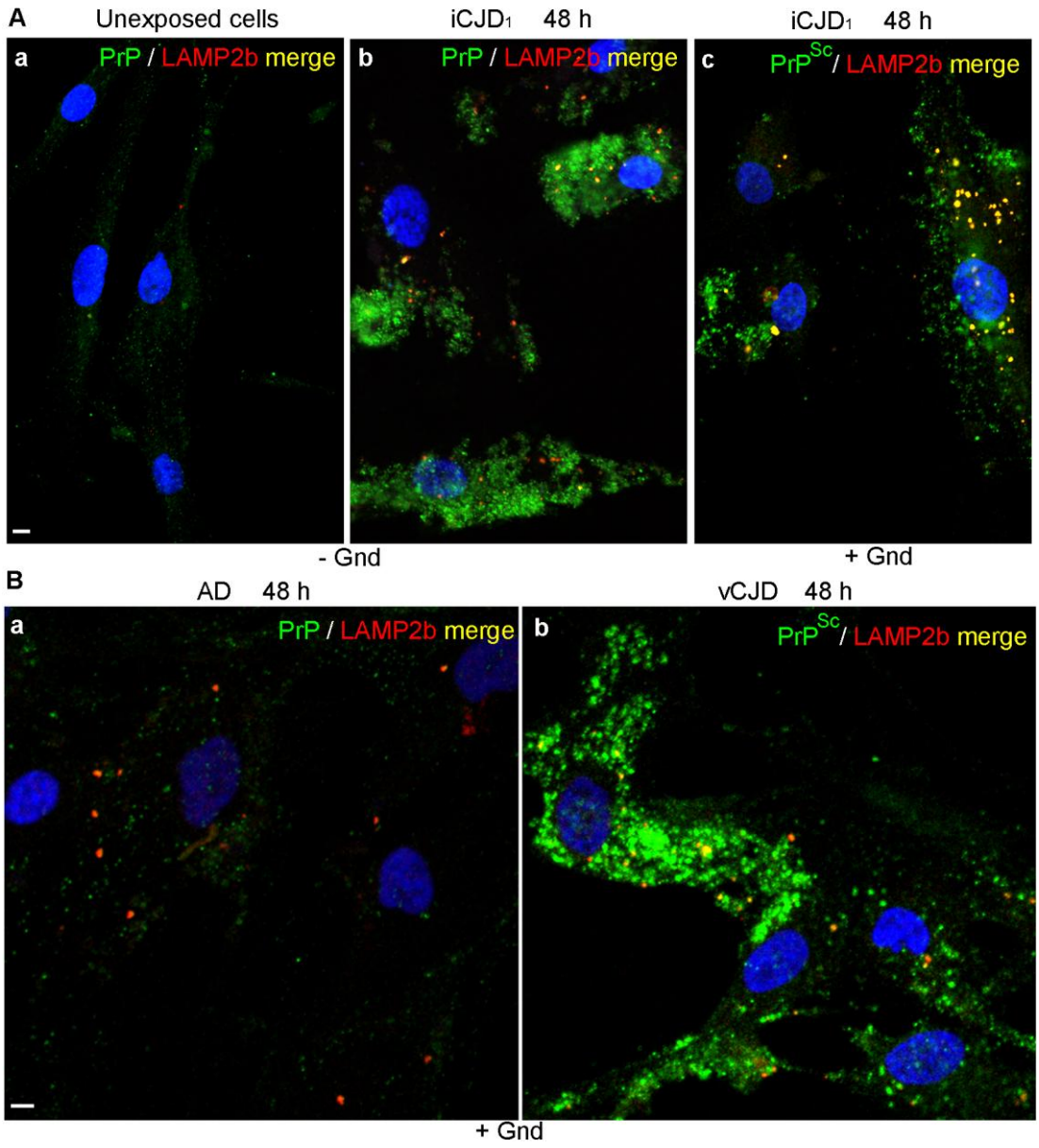


Figure 3.71: Investigation of PrP^{Sc} colocalisation with lysosomes after continuous exposure of HK cells to iCJD, vCJD and AD brain spiked medium

(A) The HK cells were grown in control medium (a) or exposed to 1% iCJD brain spiked medium for 48 hours (b, c). (B) The HK cells were continuously exposed to 1% AD (a) or vCJD brain spiked medium (b) for 48 hours. The cells were then double immunolabeled for PrP with antibody 6H4 (green) and lysosomes with LAMP2b antibody (red) without Gnd pre-treatment (Aa, Ab) or pre-treated with Gnd (Ac, Ba, Bb). The merge of the channels is shown and colocalisation of PrP^{Sc} with lysosomes is in yellow colour. The nuclei were counterstained with DAPI (blue). Scale bars, 20 µm.

3.8.3.12 Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (I.)

The next experiment was to investigate whether the results of positive colocalisation of exogenous PrP^{Sc} with lysosomes would be qualitatively similar when HK cells were immunostained after the "pulse-chase" experimental design (Figure 3.72). The cells were "pulsed" for 30 minutes at 4 °C and 15 minutes at 37 °C with either control (unspiked) complete medium (Figure 3.72; A-D) or vCJD brain spiked medium (Figure 3.72; E-H, insets of boxed areas) containing 6H4 primary antibody. Then the cells were double immunolabeled for PrP (green) and lysosomes (red) after 1 (A, E), 3 (B, F), 24 (C, G) and 48 hours (D, H) of the "chasing" period. The merge of the channels is shown. Positive colocalisation of PrP^{Sc} with lysosomes appeared as yellow. Cell-associated PrP^{Sc} was clearly detected in cells exposed to vCJD spiked medium (green). The results show the appearance of colocalisation (yellow signal) at the 48 hours time point in the cells exposed to vCJD spiked medium denoting PrP^{Sc} colocalisation within lysosomes. Therefore, the endocytosed exogenous PrP^{Sc} has most likely reached the lysosomes after 48 hours of trafficking through the exposed HK cells (Figure 3.72; H, inset).

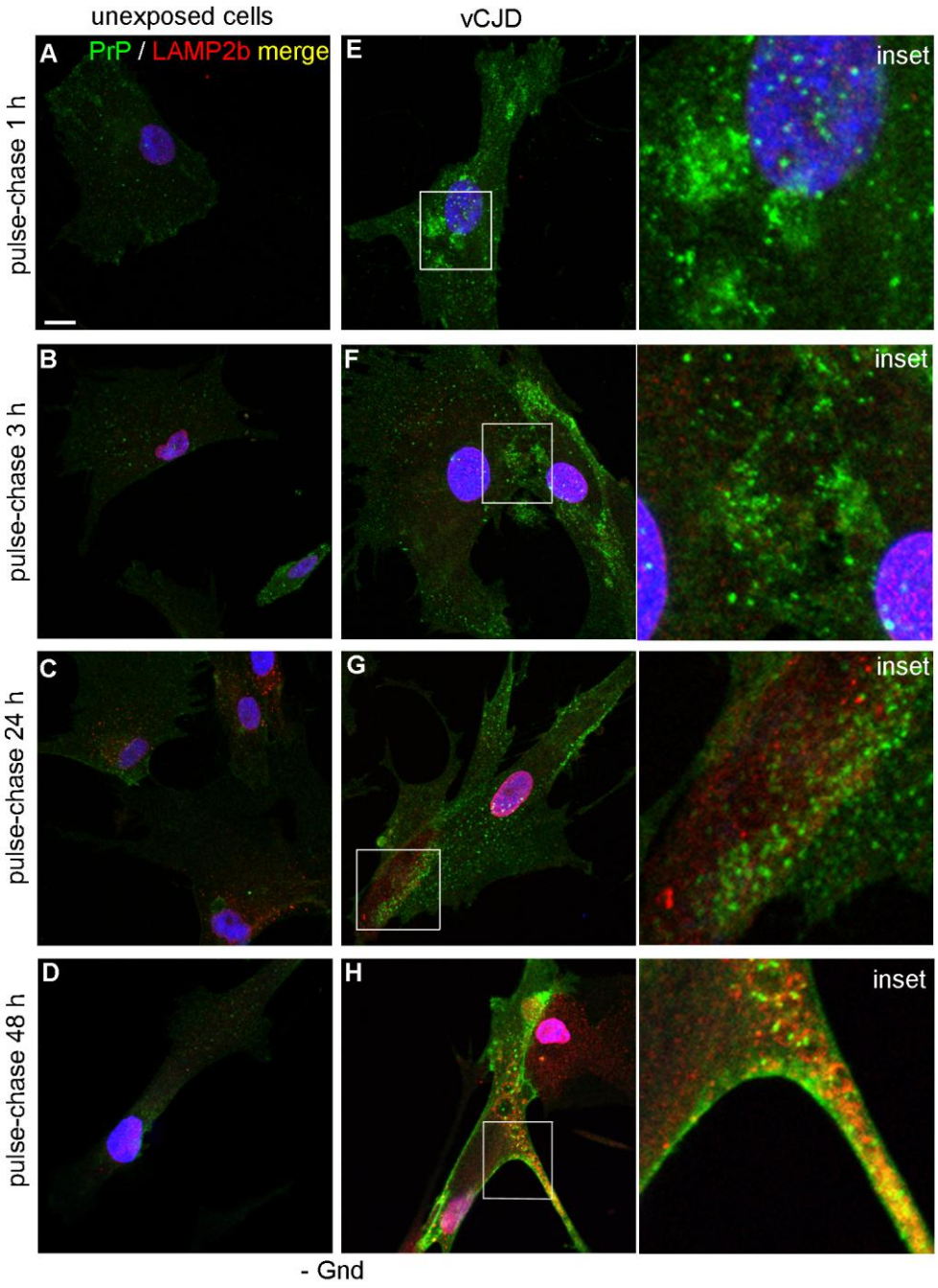


Figure 3.72: Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (I.)

HK cells were incubated with medium containing 6H4, either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and the cells were double immunolabeled for PrP (green) and lysosomes with LAMP2b antibody (red) as described in section 2.12.5. The merge of the channels is shown. Colocalisation of PrP^{Sc} with lysosomes is in yellow colour and the high magnification of the boxed areas in (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.13 Investigation of PrP^{Sc} colocalisation with lysosomes after 48 h exposure to vCJD brain material

To unambiguously demonstrate and further confirm the association of exogenous PrP^{Sc} with lysosomes, these structures were immunolabelled with another antibody used to detect lysosomes – the LAMP1 antibody (Figure 3.73).

Cells were continuously exposed to vCJD brain spiked medium for 48 hours and then double immunolabeled for PrP with the primary antibody 6H4 (green) and lysosomes with the LAMP1 antibody (red), both without guanidine pre-treatment (Figure 3.73; A) and with pre-treatment (Figure 3.73; B). Detailed display of the individual immunostaining channels is shown. The exogenous PrP^{Sc} taken up by the exposed cells was clearly demonstrated (A, B, top row). Clear evidence of exogenous PrP^{Sc} presence within the lysosomes immunolabeled with LAMP1 was observed after 48 hours exposure to the vCJD brain spiked medium (A, merged figure, inset of the boxed area and B, merged figure, inset of the boxed area). This observation further confirmed that the exogenous PrP^{Sc} is directed to lysosomal structures after being endocytosed by cells exposed to prion-spiked medium.

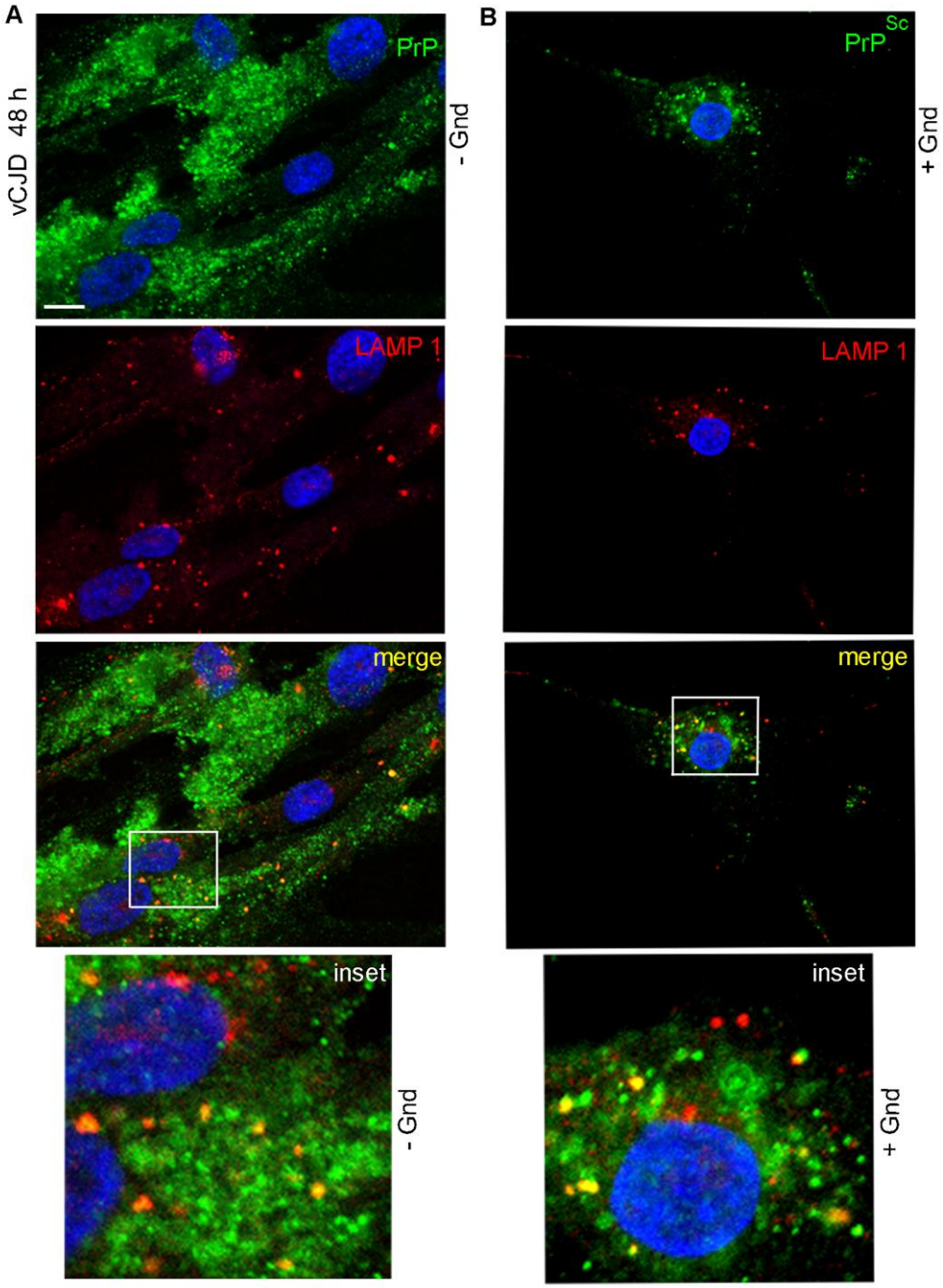


Figure 3.73: Investigation of PrP^{Sc} colocalisation with lysosomes after 48 h exposure to vCJD brain material

HK cells were continuously exposed to 1% vCJD brain spiked medium for 48 hours. The cells were then washed and double immunolabeled for PrP with antibody 6H4 (green) and lysosomes using antibody LAMP1 (red). Individual immunostaining channels are shown (A,B; top row, second row). Insets represent magnification of the boxed areas in the figures of merged channels (green and red). Colocalisation of PrP^{Sc} (A, B) in the merged channels (bottom half) is indicated in yellow. The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.14 Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (II.)

The next experiment aimed to further confirm the association of the exogenous PrP^{Sc} with lysosomes labelled with the LAMP1 antibody by the "pulse-chase" study (Figure 3.74). The design of the experiment was the same as used before and cells were analysed at routinely used time points, after being double immunolabeled for PrP (green) and lysosomes (red) with LAMP1, at 1 (A, E); 3 (B, F); 24 (C, G) and 48 hours (D, H) of the "chasing" period. Exogenous PrP^{Sc} taken up by cells exposed vCJD spiked medium was clearly shown (E-H, inset of the boxed areas). Positive colocalisation of endocytosed exogenous PrP^{Sc} with lysosomes appeared yellow. This study further confirmed previous observations that endocytosed exogenous PrP^{Sc} colocalise with cellular lysosomal structures after 24-48 hours of trafficking through cells exposed to vCJD brain material and therefore after endocytosis is most likely directed to lysosomes. Under conditions used in this study, no positive colocalisation of HK cell's own PrP^{C} with lysosomal structures could be observed in the unexposed cells (Fifure 3.74; A-D).

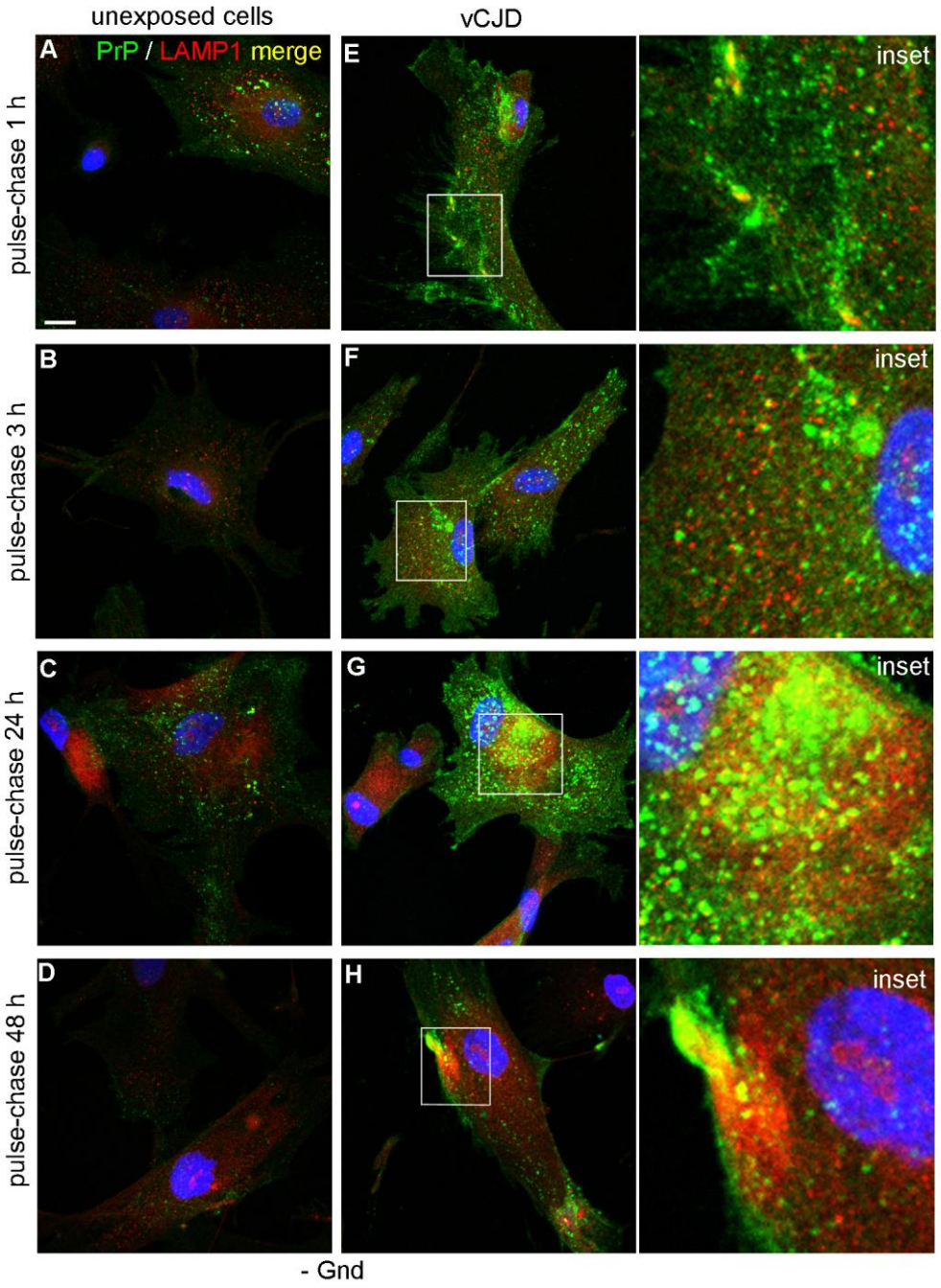


Figure 3.74: Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (II.)

HK cells were incubated with medium containing 6H4, either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and cells were double immunolabeled for PrP (green) and lysosomes using antibody LAMP1 (red) as described in section 2.12.5. The merge of the channels is shown, colocalisation of PrP^{Sc} with lysosomes is in yellow colour and insets represent magnification of the boxed areas in (E-H). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.8.3.15 Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (III.)

To unambiguously demonstrate that the PrP signal colocalising within lysosomes results from exogenous PrP^{Sc}, an additional "pulse-chase" study was performed and the immunocytochemical pre-treatment with guanidine to enhance visualisation of PrP^{Sc} was used (Figure 3.75). The merge of the channels is shown.

Indeed, the observation showed yellow clumps in the cells exposed to vCJD spiked medium at the 24-hour "chasing" period (Figure 3.75; G, inset). This observation implies that the detected PrP immunostaining signal results from exogenous PrP^{Sc} taken up by these cells. It also confirmed that the endocytosed PrP^{Sc} is trafficked to lysosomes and it reaches the lysosomal compartments approximately 24 hours after being introduced to HK cell culture. Interestingly, the colocalisation of PrP^{Sc} with LAMP1 was not apparent at the 48 hours time point.

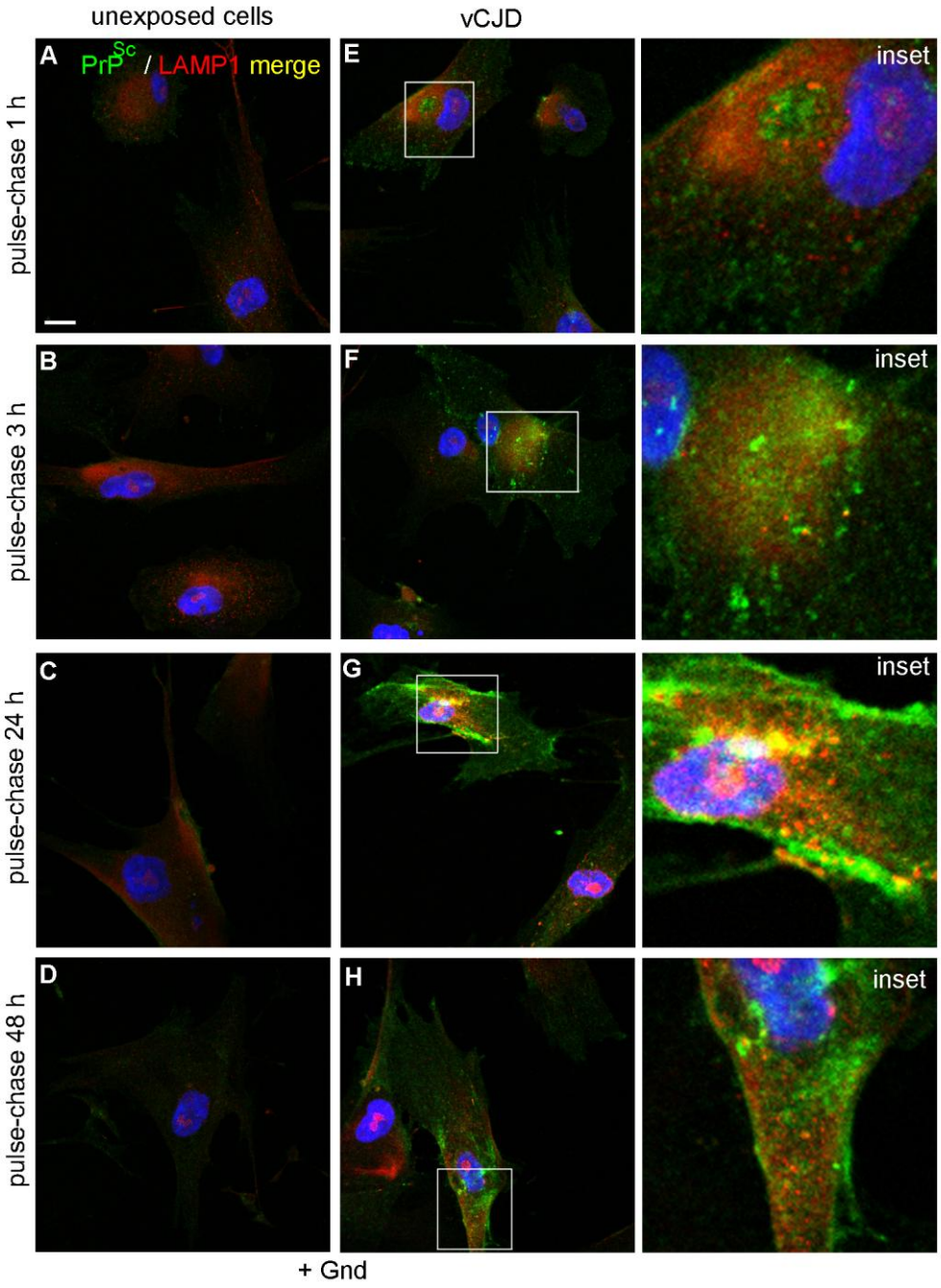


Figure 3.75: Investigation of PrP^{Sc} colocalisation with lysosomes in the "pulse and chase" study (III.)

Experimental design and treatment of the cells was the same as described before (section 3.8.14), except a guanidine pre-treatment step was included during the immunocytochemistry procedure. The cells were double immunolabeled for PrP^{Sc} (green) and lysosomes using antibody LAMP1 (red) as described in section 2.12.5. The merge of the channels is shown, colocalisation of PrP^{Sc} within lysosomes is in yellow and the high magnification of the boxed areas in (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.16 Investigation of PrP^{Sc} colocalisation with recycling endosomes in the "pulse and chase" study (I.)

As endocytosed exogenous PrP^{Sc} colocalised with lysosomal structures, a further search for an endocytic intermediate in the endosomal pathway responsible for the delivery of prion material to the lysosomes was performed.

The investigation was carried out by a "pulse-chase" study (Figure 3.76). Cells were double immunolabeled for PrP (green) and recycling endosomes (red) with the rab11A antibody at 1 (A, E); 3 (B, F); 24 (C, G) and 48 hours (D, H) of the "chasing" period. The merge of the channels is shown and positive colocalisation of endocytosed exogenous PrP^{Sc} with recycling endosomes appears yellow.

These vesicles appeared to be positive for the exogenous PrP^{Sc} at about 24 to 48 hours after the prion material has been introduced to cells (Figure 3.76; G, inset).

This experiment showed that cellular structures positive by rab11A immunostaining and resembling recycling endosomes did accumulate the endocytosed PrP^{Sc} protein.

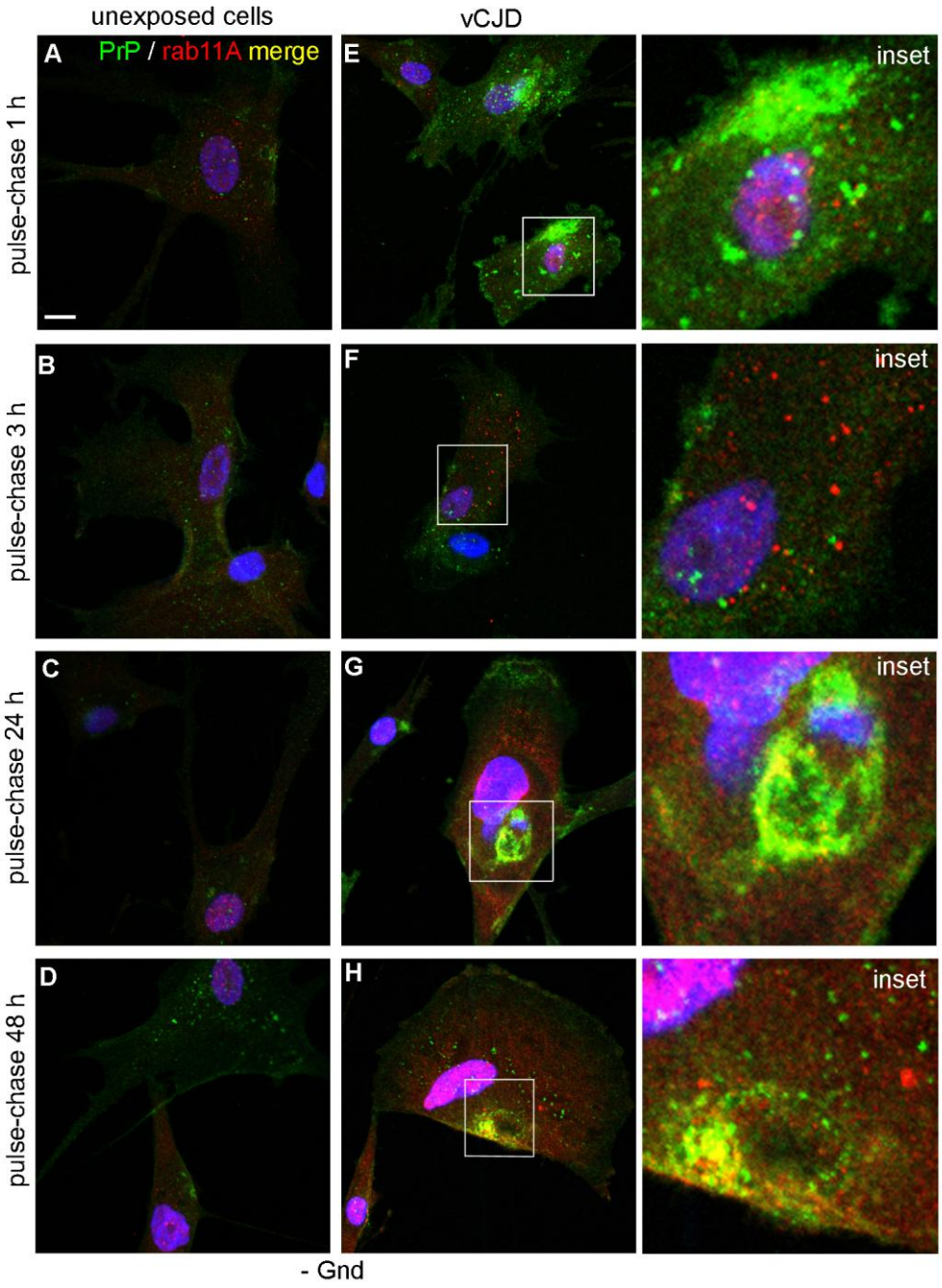


Figure 3.76: Investigation of PrP^{Sc} colocalisation with recycling endosomes in the "pulse and chase" study (I.)

HK cells were incubated with medium containing 6H4, either control (A-D) or 1% vCJD spiked (E-H) for 30 minutes at 4 °C, then for additional 15 minutes at 37 °C – "pulse". Then the cells were washed and incubated with fresh medium for 1, 3, 24 and 48 hours – "chase". The medium was then aspirated and cells were double immunolabeled for PrP (green) and recycling endosomes (red) as was described previously in 2.12.5. The merge of the channels is shown, any colocalisation of PrP and recycling endosomes is indicated in yellow colour and insets represent magnification of the boxed areas in (E-H). The nuclei were counterstained with DAPI (blue) and the scale bar represents 20 µm.

3.8.3.17 PrP^{Sc} colocalisation with recycling endosomes in the "pulse and chase" study (II.)

To demonstrate that PrP positively labelled in the recycling endosomes truly represents the exogenous PrP^{Sc} an additional "pulse-chase" study using the immunocytochemical pre-treatment with guanidine to diminish PrP^{C} staining and enhance visualisation of PrP^{Sc} was performed (Figure 3.77). Colocalisation (yellow) was observed at the 24-48 hour time point of the "chasing" period (Figure 3.77; G, H, insets). These results suggest that exogenous PrP^{Sc} is indeed present in the recycling endosomes 24-48 hours after the brain spiked material was introduced to the cultures.

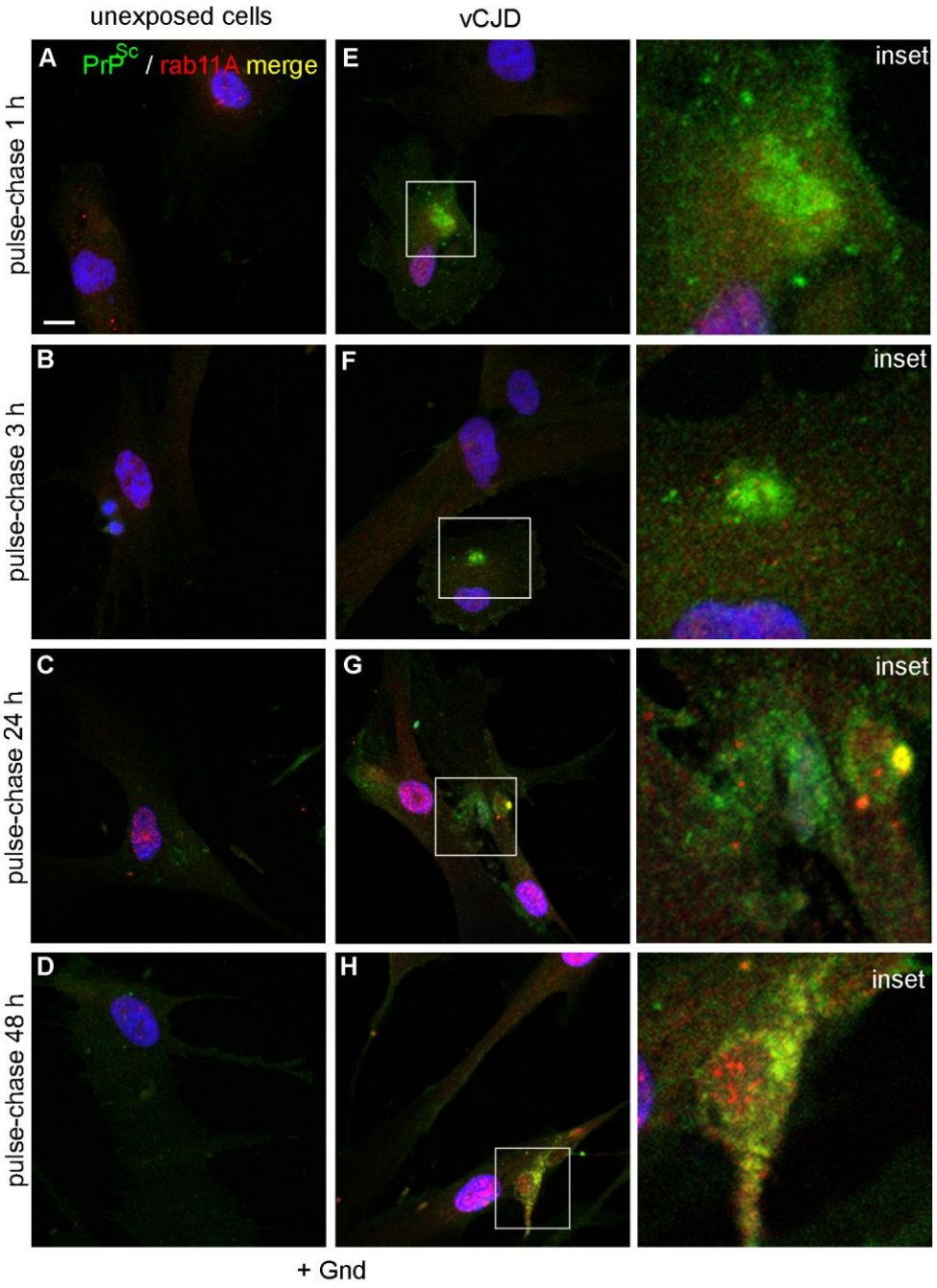


Figure 3.77: Investigation of PrP^{Sc} colocalisation with recycling endosomes in the "pulse and chase" study (II.)

Experimental design and treatment of the cells were same as described before (section 3.8.3.16) except a guanidine pre-treatment step was included during the immunocytochemistry procedure. The cells were double immunolabeled for PrP^{Sc} (green) and recycling endosomes (red) as described in section 2.12.5. The merge of the channels is shown, any colocalisation of PrP^{Sc} with recycling endosomes appeared in yellow and the high magnification of the boxed areas in (E-H) is shown right (inset). The nuclei were counterstained with DAPI (blue). Scale bar, 20 µm.

3.8.3.18 Controls for double labelling immunocytochemistry

The results of these assays were confirmed by carrying out the following controls for the immunocytochemical procedures (Figure 3.78). The combinations of antibodies tested were mixtures of the anti-PrP protein primary antibody with the secondary antibody for labelling cellular organelle antibody (A), the secondary antibody for anti-PrP protein primary antibody-FITC with the primary antibody for cellular organelle (B), anti-PrP protein primary antibody with the primary antibody for cellular organelle marker (C). The merge of the channels is shown. No signal appearance in the FITC channel confirmed that the previously observed fluorescent signal (green) was specific. Also, no signal was observed in the red channel indicating that signals in the cell organelle colocalisation studies were specific. Therefore, all PrP^{Sc} (green) colocalisation with cell organelles (red) indicated in yellow colour were considered to represent colocalisation of the antigens recognised by the primary antibodies used.

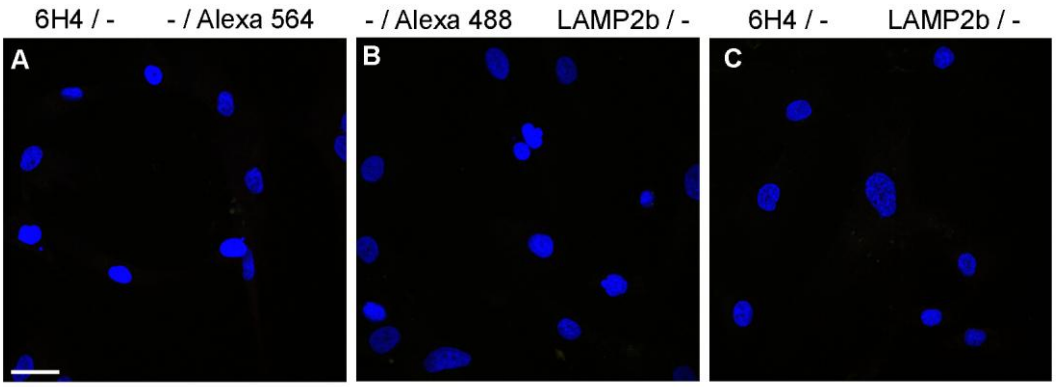


Figure 3.78: Controls of double labelling immunocytochemistry
HK cells were exposed to 1% vCJD brain spiked medium for 48 hours. Then the cells were washed, fixed, permeabilised and immunostained leaving out individual steps of adding: PrP mAb (B); FITC secondary antibody (A, C); cell organelle antibody (A); Alexa 564 (B, C). No interaction between antibodies was observed (A: mouse/rabbit anti-donkey) (B: goat-anti mouse/donkey) (C: mouse/donkey). The nuclei were counterstained with DAPI (blue). The scale bar represents 50 µm.

3.8.3.19 Quantitative image analysis and statistical assessment

In this study, two methods were used to confirm the observation that the PrP^{Sc} was truly colocalised with markers of particular intracellular structures. This was because in some circumstances the localisation of some of the cell organelle markers was widely distributed within the cell, which could appear as false positive colocalisation. The methods used were pixel-based and particle-based colocalisation analyses. They were carried out as described in detail in the Material and Methods (section 2.14). The analysis was performed on three individual images of each time point. The data are representative and correspond to observations of six independent experiments, identical in their design. The raw data of this analysis is attached (in electronic form only) as Appendix 2 of this thesis. The statistical significance of the results is summarised below in Table 3.2 and 3.3.

The pixel-based colocalisation analysis was carried out on data in which HK cells were exposed to vCJD brain homogenate and double immunolabelled for PrP^{Sc} and cell organelles: early endosomes, Golgi complex or lysosomes. The endoplasmic reticulum analysis was not carried out as the immunostaining for this cellular organelle was too faint to bear quantitative colocalisation analysis.

To assess whether the observed colocalisation was random or real the pixel values for cellular organelles (total red pixels) and PrP^{Sc} (total green pixels) were evaluated from the total pixel values (raw data as electronic Appendix 2 of this thesis). The values for random pixel colocalisation were calculated (method described in section 2.14.1). The observed pixel colocalisation was compared to the expected random pixel colocalisation. The statistical significance was assessed by considering the null hypothesis that the red and green pixels are randomly uniformly distributed. The

calculation of the P-values, the probability for the observed or greater value arising from the null hypothesis, is summarised in Table 3.2. In all cases the P-value was smaller than 2×10^{-16} (beyond the limits of the numerical accuracy of the software), indicating that the null hypothesis should be rejected (the usual significance level for rejection is 0.05 or 0.01). The anomalous P-value suggests that this method is uninformative for comparing preferential co-localisation of PrP^{Sc} with the intracellular components in the observed negative (early endosomes or Golgi complex colocalising with PrP^{Sc}) and positive results (lysosomes colocalising with PrP^{Sc}) against each other and the only conclusion that could be drawn is that the null hypothesis should be rejected. This was supported by the observation that cellular structures tend to be clustered and are unlikely to be well approximated by a uniform random distribution of pixels. Many pixel intensity-based methods (Pearson's correlation coefficient, for example) are known to suffer from similar problems (Bolte *et al.*, 2006). This also suggested that a more appropriate method for quantifying colocalisation would be to take the structure in the image into account rather than averaging over the entire image.

	Exposure time	% observed colocalisation	% expected colocalisation	P-value
EEA1	1h	1.77	0.179	less than $2x10^{-16}$
	1h	1.39	0.117	less than $2x10^{-16}$
	1h	0.87	0.207	less than $2x10^{-16}$
	24h	0.92	0.099	less than $2x10^{-16}$
	24h	2.38	0.183	less than $2x10^{-16}$
	24h	0.59	0.123	less than $2x10^{-16}$
	48h	1.32	0.079	less than $2x10^{-16}$
	48h	0.72	0.18	less than $2x10^{-16}$
	48h	0.67	0.153	less than $2x10^{-16}$
	72h	1.39	0.139	less than $2x10^{-16}$
	72h	0.63	0.113	less than $2x10^{-16}$
	72h	0.6	0.076	less than $2x10^{-16}$
Giantin	1h	0.49	0.043	less than $2x10^{-16}$
	1h	1.15	0.088	less than $2x10^{-16}$
	1h	0.71	0.064	less than $2x10^{-16}$
	24h	0.15	0.022	less than $2x10^{-16}$
	24h	0.2	0.024	less than $2x10^{-16}$
	24h	0.22	0.029	less than $2x10^{-16}$
	48h	0.53	0.043	less than $2x10^{-16}$
	48h	0.39	0.021	less than $2x10^{-16}$
	48h	0.08	0.022	less than $2x10^{-16}$
	72h	0.22	0.061	less than $2x10^{-16}$
	72h	0.45	0.042	less than $2x10^{-16}$
	72h	0.26	0.056	less than $2x10^{-16}$
LAMP2b	1h	0.1	0.004	less than $2x10^{-16}$
	1h	0.5	0.029	less than $2x10^{-16}$
	1h	0.79	0.071	less than $2x10^{-16}$
	24h	0.94	0.031	less than $2x10^{-16}$
	24h	1.13	0.027	less than $2x10^{-16}$
	24h	0.88	0.066	less than $2x10^{-16}$
	48h	0.9	0.024	less than $2x10^{-16}$
	48h	1.47	0.076	less than $2x10^{-16}$
	48h	1.53	0.106	less than $2x10^{-16}$
	72h	0.86	0.03	less than $2x10^{-16}$
	72h	0.6	0.034	less than $2x10^{-16}$
	72h	0.58	0.012	less than $2x10^{-16}$

Table 3.2: Pixel-based colocalisation analysis

Below is described the results of the analysis, based on a particle-based method of Bolte and colleagues (Bolte *et al.*, 2006), of preferential co-localisation of PrP^{Sc} with the intracellular components. The method is described in detail in the Material and Methods (section 2.14.2). The analysis was performed on the same data set as the pixel-based analysis (early endosomes and lysosomes). However, the method could not be applied to Golgi complex data as the software was unable to identify the centres of mass in such a complex morphological structures as is the Golgi complex.

The features of the software allowed calculating of a commonly used colocalisation indicator "the Manders' colocalisation coefficient" (M_1, which always has values between 0 and 1). The main goal was to evaluate the amount of the red particles (cell organelles) colocalising with the green particles (PrP^{Sc}) from the total number of present particles. The result is summarised in Table 3.3 and the raw data of this analysis are attached in electronic form as an Appendix 2 of this thesis. The confirmation of the preferential colocalisation of PrP^{Sc} with cell organelles was drawn from the values of the Manders' coefficient M_1 (red particles colocalising with green). The negative group (observed PrP^{Sc} not co-localising with early endosomes) and positive (observed PrP^{Sc} co-localising with lysosomes) are clearly distinct both in terms of the Manders' coefficient M_1 and the numbers of colocalised particles. The M_1 values are closer to value 0 in case of EEA1 (early endosomes colocalising with PrP^{Sc}) and closer to value 1 in case of Lamp2b (lysosomes colocalising with PrP^{Sc}). Also it is important to note the rising trend of the M_1 value within the Lamp2b data which correlates with increasing time of exposure of HK cells to vCJD brain homogenate and indicates a time dependent increase of lysosomes-associated PrP^{Sc}.

	Exposure time	M1	Red colocalisation with green	Green colocalisation with red	Red threshold	Green threshold
EEA1	1h	0.012	0 of 72	0 of 46	99	31
	1h	0.035	1 of 100	1 of 85	76	31
	1h	0.023	1 of 286	1 of 67	50	35
	24h	0.053	1 of 42	1 of 54	73	50
	24h	0.027	1 of 108	1 of 64	52	54
	24h	0.038	1 of 236	1 of 106	54	64
	48h	0.171	2 of 31	2 of 57	68	80
	48h	0.092	1 of 228	1 of 99	57	57
	48h	0.171	1 of 41	1 of 207	71	47
	72h	0.322	11 of 82	11 of 115	83	68
	72h	0.09	8 of 103	8 of 63	52	78
	72h	0.183	8 of 62	8 f 172	47	64
LAMP2b	1h	0.2	0 of 6	0 of 44	36	26
	1h	0.654	20 of 28	20 of 113	33	33
	1h	0.253	7 of 15	7 of 50	17	31
	24h	0.866	3 of 30	3 of 80	28	68
	24h	0.775	4 of 12	4 of 26	47	68
	24h	0.826	15 of 27	15 of 209	52	57
	48h	0.866	7 of 10	7 of 45	43	47
	48h	0.916	8 of 19	8 of 92	40	40
	48h	0.768	16 of 32	16 of 141	52	47
	72h	0.935	10 of 22	10 of 49	76	83
	72h	0.918	23 of 42	23 of 109	76	59
	72h	0.996	10 of 18	10 of 60	99	54

Table 3.3: Particle-based colocalisation analysis

3.8.4 Summary

- After introducing the prion diseased brain material to HK cells, PrP^{Sc} uptake and its dynamical transport to the perinuclear region was observed as in earlier studies (3.3-5).
- The HK cells appeared to show trafficking of exogenous PrP^{Sc} to recycling endosomes and then to lysosomal structures.
- Roles for the Golgi complex and the endoplasmic reticulum in exogenous PrP^{Sc} trafficking were excluded.
- However, some yellow particles indicating positive colocalisation of the exogenous PrP^{Sc} with early endosomes was observed at the 24 hours time point of continuous exposure and the 3-48 hours time point of the "pulse-chase" study. That this colocalisation was random was comfirmed by a quantitative image analysis study in this section.
- The endosomal recycling compartment and lysosomes were identified as the likely sites of prion intracellular trafficking and degradation. This phenomenon could be observed both in the continuous exposure and in the "pulse-chase" studies.
- PrP^{Sc} colocalisation with the LAMP1 and LAMP2b antibodies gave a closely similar pattern in cells continuously exposed to brain homogenate and in the "pulse-chase" studies, indicating that the vCJD PrP^{Sc}-6H4 complex did not change or delay the trafficking of exogenous PrP^{Sc} into lysosomes.
- The exogenous PrP^{Sc} was shown structures positively immunolabeled with the rab11A antibody, resembling recycling endosomes, after 24 hours

trafficking through the cell. At the same time, it was also observed within the lysosomal structures.

4. DISCUSSION

The initial aims of this thesis were to characterise two different non-neuronal cell types in respect of their prion protein genetics, expression level and cell biology and subsequently to determine whether these cells could be infected with selected human prions after exposure to post-mortem human brain homogenates. The findings of this study are discussed in relation to what is beginning to be learnt about the cell biology and trafficking of normal and abnormal PrP^{Sc} and how this might relate to cellular susceptibility to prion infection and to cytotoxicity.

4.1 PRP AND CYTOTOXICITY

4.1.1 Is PrP^{Sc} cytotoxic?

The *Prnp* gene that encodes PrP^{C} was identified in 1985 (Oesch *et al.*, 1985; Basler *et al.*, 1986) and the first *Prnp* knockout mice were established in 1992 (Bueler *et al.*, 1992). However, the function of the prion protein is not known with certainty. In spite of the extensive research the molecular mechanisms that result in neuronal damage, neuronal dysfunction and ultimately widespread neuronal loss in the brain in prion diseases remain poorly understood (Wilson, 2005; Wilson *et al.*, 2007). There is a growing consensus that neuronal damage does not result from a loss of functional PrP^{C}, because its depletion is not detrimental and does not trigger any gross pathology in various experimental settings. For example, PrP^{C} is essential for propagation of infectious prions and neurotoxicity (Bueler *et al.*, 1993), PrP^{C} knockout in the adult mouse brain does not result in overt phenotypic anomalies

(Mallucci *et al.*, 2002) and embryonic PrP^{C} knockout models exhibit normal development (Bueler *et al.*, 1992; Manson *et al.*, 1994). Based on those observations, PrP^{C} knockout studies argue against the loss of PrP^{C} function in neurones as a substantial mechanism in prion-mediated neurodegeneration. However, the possibility of compensation for the depleted PrP^{C} by a shadoo protein has been proposed (Watts and Westaway, 2007).

A toxic gain of function by PrP^{Sc} is an attractive alternative hypothesis. The neurotoxicity of PrP^{Sc} aggregates is debatable (Lansbury and Lashuel, 2006). Both full-length PrP^{Sc} (Hetz *et al.*, 2003; Novitskaya *et al.*, 2006) but also shorter PrP^{Sc} peptides (Forloni *et al.*, 1993) have been proposed to be toxic to cells *in vitro*, but the relevance of their toxicity *in vivo* is not clear. Several lines of evidence suggest that PrP^{Sc} may not be the toxic species. PrP-null tissue can be in the vicinity of PrP^{Sc} deposits without suffering deleterious effects (Brandner *et al.*, 1996a; Mallucci *et al.*, 2003), and no direct correlation between neuronal loss and PrP^{Sc} plaques in the brains of individuals affected with sCJD can be demonstrated (Parchi *et al.*, 1996; Kristiansen *et al.*, 2005; Kong and Bessen, 2008; Deriziotis and Tabrizi, 2008). Furthermore, cases of prion diseases (FFI, VPSPr) in which disease associated PrP is hardly detectable after PK digestion (termed PrP^{sen}) were reported (Medori *et al.*, 1992b; Parchi *et al.*, 1999a; Gambetti *et al.*, 2003; Gambetti *et al.*, 2008; Head and Ironside, 2009; Jansen *et al.*, 2010; Zou *et al.*, 2010; Head *et al.*, 2010), examples of infectivity in the absence of detectable PrP^{Sc} have been described (Collinge *et al.*, 1995; Wille *et al.*, 1996; Lasmezas *et al.*, 1997; Shaked *et al.*, 1999) and transgenic mouse models have shown that several aberrant conformers of PrP distinct from PrP^{Sc} can evoke neuronal cell death in the absence of infectious prion propagation

(Muramoto *et al.*, 1997; Chiesa *et al.*, 1998; Hegde *et al.*, 1998; Shmerling *et al.*, 1998; Ma *et al.*, 2002; Flechsig *et al.*, 2003; Baumann *et al.*, 2007). Moreover, subclinical infection, in which high levels of PrP^{Sc} accumulate in the absence of clinical symptoms have also been described (Hill *et al.*, 2000; Race *et al.*, 2001; Race *et al.*, 2002; Hill and Collinge, 2003; Piccardo *et al.*, 2007). In addition, prion-infected mice expressing PrP^{C} lacking the GPI anchor, were shown to produce infectious prions followed by accumulation of extracellular PrP amyloid plaques, but surprisingly did not succumb to disease (Chesebro *et al.*, 2005; Trifilo *et al.*, 2006). These data thus argue against accumulated PrP^{Sc} being the cytotoxic species.

4.1.2 Subcellular localisation and topology

Targeting of PrP to the cytosol was shown to cause a rapidly lethal neurodegeneration, although accumulation of PrP^{Sc} was not detected (Ma *et al.*, 2002; Chesebro *et al.*, 2005; Trifilo *et al.*, 2006; Rane *et al.*, 2008; Rane *et al.*, 2010). Moreover, proteasome inhibition *in vitro* was shown to induce formation of a slightly-protease resistant cytoplasmic PrP form in cultured cells (Ma and Lindquist, 2002). Based on these findings, prion toxicity was proposed to initiate with retrotranslocation of PrP^{C} from the endoplasmic reticulum to the cytosol as a consequence of impaired proteasomal function. Subsequent studies have shown that cytosolic PrP (cyPrP) retains its secretory signal peptide and moreover is devoid of a GPI anchor, indicating that it cannot enter the endoplasmic reticulum (Drisaldi *et al.*, 2003).

The basic phenomenon of toxicity of cytosolic PrP has been contested (Roucou *et al.*, 2003; Heller *et al.*, 2003; Fioriti *et al.*, 2005). Lingappa and colleagues proposed that

PrP^{C} can assume two different endoplasmic reticulum trans-membrane topologies (^{Ctm}PrP transmembrane PrP with an extracellular C-terminus and ^{Ntm}PrP transmembrane PrP with an extracellular N-terminus), where ^{Ctm}PrP concentrations was shown to correlate with neurotoxicity and ^{Ctm}PrP was suggested to represent a major toxic moiety (Hegde *et al.*, 1998; Hegde *et al.*, 1999). However, these observations are controversial, with evidence both for (Ma *et al.*, 2002; Heller *et al.*, 2003; Rane *et al.*, 2004; Wang *et al.*, 2005; Rambold *et al.*, 2006) and against (Roucou *et al.*, 2003; Drisaldi *et al.*, 2003; Fioriti *et al.*, 2005) this neurotoxic consequences of cytoplasmic PrP^{C} accumulation.

4.1.3 Toxic intermediates or by-products

A recent study by Collinge and co-authors has again raised the important point that the infectious and toxic forms of PrP need not be identical (Sandberg *et al.*, 2011). The gain of toxicity by a PrP moiety, other than conventional PrP^{Sc}, remains a distinct possibility. It has been proposed that during conversion of PrP^{C} to PrP^{Sc}, a toxic intermediate may be produced (Safar and Prusiner, 1998; Hill *et al.*, 2000; Tzaban *et al.*, 2002; Caughey and Lansbury, 2003; Haass and Selkoe, 2007; Thackray *et al.*, 2007; Godsave *et al.*, 2008; Cronier *et al.*, 2008; Sanghera *et al.*, 2008; Pirisinu *et al.*, 2010; D'Castro *et al.*, 2010; Sandberg *et al.*, 2011; Arellano-Anaya *et al.*, 2011). This intermediate or toxic side product has been termed PrP^{L} by Collinge and co-workers (Hill and Collinge, 2003; Kristiansen *et al.*, 2007; Collinge and Clarke, 2007). This model considers PrP^{Sc} as a moderately inert end-product, whereas the steady state level of PrP^{L} determines the rate of neurodegeneration (Hill and Collinge, 2003; Sandberg *et al.*, 2011). It predicts that PrP^{L} levels are regulated

by natural clearance mechanisms and its accumulation occurs only when PrP^{L} overwhelms the clearance capacity, then resulting in neurodegeneration.

A proposed candidate for such a toxic species is a soluble monomeric or oligomeric conformer of the prion protein (Hill and Collinge, 2003). It is unclear how PrP^{L} might relate to the "silent prions" demonstrated biochemically in normal human brains by Gambetti and colleagues (Yuan *et al.*, 2006).

4.1.4 PrP^{C} signalling

Tatzelt and colleagues have demonstrated that PrP^{C} localised at the cell surface is able to mediate toxic signalling, without being converted into PrP^{Sc} by interaction with β-sheet-rich conformers and that this event is dependent on the N-terminal domain of PrP^{C} and the C-terminal GPI anchor (Solforosi *et al.*, 2004; Resenberger *et al.*, 2011). The data is apparently in conflict with previous findings that PrP^{C} can activate neuroprotective signalling pathways (Kuwahara *et al.*, 1999; Brown *et al.*, 1999; Bounhar *et al.*, 2001; Chiarini *et al.*, 2002; McLennan *et al.*, 2004; Shyu *et al.*, 2005; Spudich *et al.*, 2005; Weise *et al.*, 2006; Mitteregger *et al.*, 2007; Rangel *et al.*, 2007) and that the C-terminal GPI anchor and the N-terminal domain are essential for this physiological activity (Rambold *et al.*, 2006; Mitteregger *et al.*, 2007). Thus, it could be concluded that PrP^{C} acts as a signalling molecule at the cell surface to promote stress-protective signalling under physiological conditions, which can be switched to toxic signalling initiated by interaction with β-sheet-rich conformers and that PrP^{C} most probably acts as a co-receptor in concert with a transmembrane protein to transduce the signal into the cell (Winklhofer and Tatzelt, 2000; Flechsig and Weissmann, 2004; Roucou and LeBlanc, 2005; Resenberger *et al.*, 2011).

4.1.5 PrP^{Sc} uptake

The proposal that accumulation of PrP^{Sc} in the cytosol of chronically infected cells is generally innocuous *in vitro* (Haigh *et al.*, 2011) is in close agreement with observations in this thesis (section 3.5). The two distinct non-neuronal cell systems used in this study - the FDC-like HK cells and undifferentiated hESC - showed uptake and substantial accumulation of exogenous PrP^{Sc} from a medium spiked with prion disease brain homogenates. No difference in signal between cells exposed to brain material and control cells in live cells staining – as opposed to a notable difference between exposed and control cells in fixed and permeabilised cells - indicates that the PrP^{Sc} signals were indeed intracellular rather than material from the medium deposited or trapped on the surface. The cells displayed strong intracellular accumulation of PrP^{Sc} in juxtanuclear region of the cytosol with a coarse granular morphology. Examination of these cultures did not reveal any gross changes in cell morphology, growth characteristics or viability even after continuous incubation with the brain spiked medium for 72 hours and when the cells exhibited substantial intracellular accumulation of PrP^{Sc}.

The fact that these cells do not support prion infection and apparently represent accumulation of PrP^{Sc} in the cytosol without toxic results is consistent with prion toxicity being independent of PrP^{Sc} accumulation in the cell. This observation is not unique to the cell types used in this thesis. Similar localisation (prominent juxtanuclear staining) can be seen in other studies involving prion infected cells (Mange *et al.*, 2004; Kristiansen *et al.*, 2005; Magalhaes *et al.*, 2005; Marijanovic *et al.*, 2009; Veith *et al.*, 2009).

Collectively, these data support the proposal that large aggregates of PrP^{Sc} are neither the causal reason nor sufficient for cytotoxicity and the development of prion

infection. Instead, attention has turned to soluble (Silveira *et al.*, 2005; Berardi *et al.*, 2006), more protease-sensitive (Tzaban *et al.*, 2002; Pastrana *et al.*, 2006), and small oligomeric forms of the prion protein (Silveira *et al.*, 2005; Novitskaya *et al.*, 2006; Kristiansen *et al.*, 2007; Simoneau *et al.*, 2009) as being responsible for toxicity.

Cell lines susceptible to prion infection and capable of propagating infection seemingly without adverse effects have proved valuable for TSE research. Recent studies have questioned whether such an infection is free of adverse effects, even when the cells appear phenotypically normal (Haigh *et al.*, 2011). This supports an earlier report by Schatzl and collaborators who demonstrated that the GT1-7 cell line exhibits cytopathology in approximately 20% of cells chronically infected with scrapie derived RLM prion strain (Schatzl *et al.*, 1997). The apparent discrepancy between effects of *in vivo* and *in vitro* PrP^{Sc} formation may perhaps also be due to the transformed phenotype of the cell culture models used. This could mask the PrP^{Sc} neurotoxicity, which may manifest itself only in terminally differentiated cells, resembling more the post-mitotic phenotype of the central nervous system neurons. This may explain why ScGT1 cells, with a more differentiated neuronal phenotype, do exhibit signs of neurotoxicity, whereas cells of other phenotypes (eg. ScN2a or ScN1E-115) do not. Such cells may therefore offer greater possibilities for the study of prion related toxicity and pathogenesis than is currently thought, allowing the identification of low-level, but ongoing, cell damage. Alternatively, the formation and/or accumulation of PrP^{Sc} may not be neurotoxic *per se* but rather as "neuro-stressant" and perhaps requiring interplay with microglia and/or astrocytes to become fully neurotoxic.

4.1.6 Infection studies

The work described in this thesis directly examines the cellular response of extraneuronal lymphoreticular FDC-like HK cells and undifferentiated human embryonic stem cells to exposure to infectious prions from human (variant, sporadic, iatrogenic Creutzfeldt-Jakob disease) and animal (bovine spongiform encephalopathy) brains and their susceptibility and ability to propagate prions *in vitro*. Susceptibility-and in some instances, disease phenotype-in human prion diseases are known to be influenced by the methionine/valine (MV) polymorphism at codon 129 of the *PRNP* gene (Collinge and Palmer, 1991; Goldfarb *et al.*, 1992; Monari *et al.*, 1994; Parchi *et al.*, 1996; Zeidler *et al.*, 1997; Deslys *et al.*, 1998; Lee *et al.*, 2001; Brandel *et al.*, 2003). Therefore, the analysis of *PRNP* codon 129 polymorphism, as well as the level of PrP^{C} expression of the cell lines chosen for these studies, was an important consideration (section 3.1).

The chosen human embryonic stem cell lines were of the MM (RCM-1) and MV (RH1) genotypes at codon 129 of *PRNP*. Unfortunately, the only line with a VV genotype within the seven available hESC lines had poor growth characteristics and therefore was not used. The available follicular dendritic cell-like cell line (HK) was found to be VV. The level of PrP^{C} expression tested before the actual infection studies was found to be in the hESC RCM-1 and RH1 lines below the detection limit by Western blot, whereas there was abundant PrP^{C} expression by the FDC-like HK cells. An initial interest was to test whether the cell lines had all the required components to support replication of PrP^{Sc} *in vitro*. This was carried out by PrP^{Sc} amplification in cell-free assay (PMCA). Cell extracts of established cell lines of neuronal or non-neuronal origins were successfully used as a substrate source for PMCA only very recently (Mays *et al.*, 2011). This was in spite of earlier reports that

indicated that a cell culture derived substrate is incapable of supporting PrP^{Sc} formation in PMCA unless complemented with brain homogenate that may include essential cofactors for PrP conversion (Saborio *et al.*, 1999; Castilla *et al.*, 2006).

The data gained in this experiment (section 3.3) confirmed previous observation that the abundance of PrP^{C}, this case in cell lysate, is a critical factor for successful PrP^{Sc} conversion (Bueler *et al.*, 1993). Therefore, it is also a critical factor to drive efficient PrP^{Sc} amplification in a cell-free system (Mays *et al.*, 2011). PMCA amplification of PrP^{Sc} using the extracts from hESC was not successful (data not shown). Cell exctracts from HK cells were capable of supporting PrP^{Sc} formation and therefore these cells were considered to have the essential minimal requirements to support prion replication *in vitro*. The ability of HK cell lysate to support PrP^{Sc} formation was comparable in efficiency to that of normal brain material under the same conditions and demonstrated that cell lysate is sufficient to support PrP^{Sc} amplification in a *PRNP* codon 129-depended manner. However, it has been shown previously that host encoded factors other than PrP^{C} may also be required to propagate prions *in vitro* and *in vivo* (Telling *et al.*, 1995; Saborio *et al.*, 1999; Stephenson *et al.*, 2000; Lloyd *et al.*, 2001; Manolakou *et al.*, 2001; Deleault *et al.*, 2007). The restricted range of cell types that are susceptible to prion infection also suggests the existence of prion propagation co-factors (Raeber *et al.*, 1999; Bosque and Prusiner, 2000; Enari *et al.*, 2001).

These observations could be taken to suggest that PMCA might be viewed as a rapid indicator of a cell type's potential susceptibility to prion infection *in vitro* and *in vivo*. This proposition was tested by directly challenging HK cells *in vitro* with some of the brain homogenates used to seed the above PMCA reactions. The cell types

likely to be involved in propagations of prions *in vivo* were proposed to be low density (Clarke and Kimberlin, 1984), long-lived and mitotically quiescent cells (Fraser and Farquhar, 1987; McBride *et al.*, 1992). Follicular dendritic cells (FDCs) express PrP^{C} and are suggested to be a major site of prion replication and accumulation in the germinal centres of spleen, lymph nodes and mucosa-associated lymphoid tissue following experimental or natural infection with prions (Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Klein *et al.*, 1998; Hill *et al.*, 1999; Sigurdson *et al.*, 1999; Andreoletti *et al.*, 2000; Beekes and McBride, 2000; Mabbott *et al.*, 2000; Heggebo *et al.*, 2002; Mabbott *et al.*, 2003). However, these cells may not account for the entire process of prion neuroinvasion on its own as they are known to generate immobile networks. Although nerve ending within the lymphoreticular system tissues may provide one route to neuroinvasion (Glatzel *et al.*, 2001; Aguzzi *et al.*, 2003; Glatzel *et al.*, 2004), macrophages were also suggested to be able to take up infectious prions and sequester infectivity (Carp and Callahan, 1981), and dendritic cells may also be responsible for the spread of prions throughout the body (Aucouturier *et al.*, 2001; Huang *et al.*, 2002) as they display potential mobility and are known to have a close contact with macrophages (Carp and Callahan, 1981; Beekes and McBride, 2000; reviewed by Koperek *et al.*, 2002; Beekes and McBride, 2007; revieved by Kovacs and Budka, 2008).

The observation that the HK cells employed in this thesis share characteristics with FDC cells (Kim *et al.*, 1994; Kim *et al.*, 1995), express PrP^{C} and support PrP^{Sc} replication using their extracts in cell-free system, might be taken to suggest that HK cells should support PrP^{Sc} propagation *in vitro*. The cellular response of the HK cells and the hESC to acute exposure of infectious prions was tested. This was monitored

by Western blot analysis (section 3.4) and also by immunocytochemical analysis (sections 3.6). The HK cells and hESC exposed to a medium containing prion diseased brain homogenate progressively take up PrP^{Sc} (and presumably prion infectivity) present in the medium. The amount of cell-associated PrP^{Sc} increased with the time of exposure to the spiked medium. The uptake of PrP^{Sc} was rapid, and the estimation of total PrP^{Sc} uptake into the cells for each given time point showed that there were no obvious differences in PrP^{Sc} uptake between the cell types when using different brain homogenates.

However, membrane binding and internalisation of exogenous (inocula-derived) PrP^{Sc} may not be sufficient to generate prion infection and PrP^{Sc} production in itself, and other cellular factors, in addition to a simple physical interaction between PrP^{C} and PrP^{Sc}, may be required (Deleault *et al.*, 2007; Abid *et al.*, 2010; Goold *et al.*, 2011). Some previous studies have proposed that as little as four minutes of exposure to prions is sufficient for the cell to become infected and distribute PrP^{Sc} at steady-state in the perinucleus and cell surface (Caughey and Raymond, 1991; Arnold *et al.*, 1995; Kristiansen *et al.*, 2005). A recent publication indicated that only two minutes of exposure is sufficient to generate cells that continue producing PrP^{Sc} in the absence of a further prion seed. These studies appear to show that cells can be stably infected with prions following a very transitory exposure (Goold *et al.*, 2011).

Despite the abundant expression of PrP^{C}, the ability to support PrP^{Sc} replication using the cell extracts in a cell-free system, and the rapid uptake and intracellular accumulation of PrP^{Sc} in the cytosol after exposure to prion diseased brain material, the HK cells did not show any signs of prion replication and accumulation *in vitro* (section 3.5). A wide variety of inoculum and exposure protocols were employed:

usage of different concentrations of brain homogenates, sonication, homogenisation in physiological buffers, or even in non-ionic detergents, and culturing the cells in low nutritional conditions or in medium without antibiotics, but none were successful. The conditions for cell challenge were largely based on previous successful prion infections of other cultured cell types. Sonication of brain homogenates before cell challenge seemed to facilitate better internalisation and trafficking, suggesting that size of exogenous PrP^{Sc} aggregates strongly influences the internalisation rate (Magalhaes *et al.*, 2005). PrP^{C} is mainly present in rafts (cholesterol rich domains) in the cell membrane and drugs that interfere with the raft biology could, in principle, impair PrP^{C} or PrP^{C}/PrP^{Sc} complex endocytosis and thus delay or prevent PrP^{Sc} accumulation in treated cells (Mange and Lehmann, 2002; Dormont, 2003). Because Amphotericin B (present in the cell culture antibiotic/antimycotic cocktail) can inhibit PrP^{Sc} generation in scrapie-infected GT1-7 and N2a cells by modifying properties of the detergent-resistant microdomains (Mange *et al.*, 2000a; Mange *et al.*, 2000b), it was also decided to test the HK cell susceptibility to prion infection when challenged with a medium containing infectious prions without the antibiotic cocktail. Other studies inclined to expose cells to PrP^{Sc} homogenised in physiological buffers, arguing that PK-digested forms might have poor biological relevance.

Despite numerous attempts, using a series of rationally designed methodological variations, PrP^{Sc} was internalised, but no evidence was found for HK cell infection or prion replication. This was a disappointing finding, especially since efforts were made to avoid transmission barriers based on species and codon 129 genotype. It was especially surprising that iatrogenic CJD (VV) prions failed to infect the HK cells,

which abundantly expressed PrP^{C} 129 VV, but this could perhaps be attributed to the sCJD/iCJD prion strain being neurotropic rather than lymphotropic.

The most likely explanation for the PrP^{res} signal observed in Western blots of the cell lysates from cells exposed to prion disease brain material at early time points is that it represents a residual inoculum, which was then lost or diluted out during longer term culturing and subsequent passages. Analysis using the 3F4 and 6H4 antibodies in cells exposed to BSE is consistent with this explanation. However, it cannot be ruled out completely that some HK cells may exhibit a transitory or perhaps fatal prion infection within the exposed cultures as a whole. Judged by the gradual uptake and subsequent loss of PrP^{Sc} in the cells exposed to prion infectious medium and then cultured in fresh medium, it appears that the PrP^{Sc} was most likely actively internalised and then degraded by the cells, rather than being non-specifically bound to the cell surface. Interestingly, the cell-associated PrP^{Sc} signal appeared to be more intense in first hours of recovery than in the cell lysate just before the removal of infectious medium. Overall, these data lead to the conclusion that HK cells are largely resistant to infection with human and bovine prions or that they are not competent to sustain an infection.

Similarly, it was perhaps surprising that 129 MM hESC failed to become infected with BSE or vCJD. It is possible that this was a reflection of the very low level of PrP^{C} expression by the self-renewing populations of hESC used. The fact that both the HK cells and the hESC grow and divide may be relevant, as healthy uninfected cells may outgrow any rare infected, and perhaps less healthy, cells. It is noteworthy that the main site of prion replication is in a post-mitotic cell, such as neuron or FDC. Alternatively, perhaps neither hESC nor HK cells express the correct hypothetical

co-factors or the appropriate sub-cellular milieu for efficient conversion. It is worth noting that although human FDCs *in situ* show signs of infection (in 129 MM individuals with primary and secondary vCJD), HK cells and FDC differ in several important and relevant respects. FDC *in situ* are largely post-mitotic and have functional relationships with surrounding cells in secondary lymphoid cells. FDC also have elaborate projections, a highly specialised cell surface, and part of their function in the immune response involves the trapping and long-term maintenance, rather than endocytosis and degradation of antigens on the cell surface.

Whether acute but transitory *de novo* PrP^{Sc} formation can be initiated in these cells without leading to sustainable PrP^{Sc} formation merits further investigation as does the mechanism by which cell-associated PrP^{Sc} was lost during this process. The latter was further investigated in this thesis (sections 3.6 and 3.8).

4.2 CELL BIOLOGY AND PRP TRAFFICKING

4.2.1 Uptake and endocytosis

- The trafficking of molecules within cells is most likely dependent on the mechanism of internalisation (Conner and Schmid, 2003). The relevant data currently in the literature largely concerns internalisation of PrP^{C} or PrP^{Sc} in the context of constitutively infected cells, and not naïve cells exposed to exogenous prions. Undifferentiated hESC cells and HK cells display a readily detectable time dependent uptake of PrP^{Sc} from medium spiked with prion disease brain homogenates (sections 3.4, 3.5, 3.7, 3.8). Densitometric assessment (3.4) confirmed the rising trend of the cell-associated PrP^{Sc} signal increasing with the time of exposure of the cells to the brain spiked medium.

The presence, distribution, and time course of uptake of exogenous PrP^{Sc} by hESC and HK cells was similar, whether they were exposed to BSE, vCJD, iCJD, or sCJD brain homogenates. Moreover, the *in vitro* experimental evidence presented here is in agreement with previous observations that PrP^{C} is not required for internalisation of PrP^{Sc} (Vey *et al.*, 1996; Paquet *et al.*, 2007; Dorban *et al.*, 2010). Section 3.1 demonstrated that the HK cells express abundant level of PrP^{C} whereas the undifferentiated hESC express levels below detection by Western blot.

Nevertheless, the amount of PrP^{Sc} taken up and accumulated by both cell types was similar. Furthermore, no differences in the rate of uptake were observed, which could be attributed to differences of cell type. Overall it was concluded that uptake is neither dependent on the species origin of the brain homogenate, nor the *PRNP* codon 129 genotype of the cell or the prion agent, and that PrP^{C} is not necessary for internalisation of PrP^{Sc}.

Endocytosis is known to provide a crucial and dynamic interference between the extracellular milieu and the interior of the cell (Mayor and Pagano, 2007; Idone *et al.*, 2008). To investigate the precise mechanism HK cells used for internalisation of the exogenous PrP^{Sc}, immunocytochemical procedures investigating PrP^{Sc} colocalisation with endocytic vesicles visualised by confocal microscopy were employed (section 3.7).

Employing guanidine pre-treatment and continuous exposure or the "pulse-chase" paradigm provided direct evidence that both caveolae and clathrin-mediated endocytic pathways are involved in the process of exogenous PrP^{Sc} uptake. Interestingly, the data presented here indicates that caveolae-coated vesicles are the major early route for PrP^{Sc} uptake. The evidence presented here further suggests that

the kinetics of uptake differ, with caveolae-mediated uptake preceding that of clathrin coated pits. These data are consistent with reports for constitutively infected cells in which PrP^{Sc} internalisation is caveolae-mediated (Vey *et al.*, 1996; Wadia *et al.*, 2008; Jen *et al.*, 2010). Although PrP^{C} recycling was not studied in this thesis, endocytosis, via clathrin-coated pits and via caveolae-coated vesicles have been separately implicated by different researchers (Ying *et al.*, 1992; Shyng *et al.*, 1993; Shyng *et al.*, 1994; Shyng *et al.*, 1995; Vey *et al.*, 1996; Marella *et al.*, 2002; Sunyach *et al.*, 2003; Peters *et al.*, 2003; Griffiths *et al.*, 2007; Sarnataro *et al.*, 2009). The reported discrepancies in these studies may result from different approaches, cell types and experimental paradigms employed. Nevertheless, the endocytic pathway has been proposed to be important for the internalisation and conversion of PrP^{C} to PrP^{Sc} (Borchelt *et al.*, 1992; Vey *et al.*, 1996; Kaneko *et al.*, 1997a; Campana *et al.*, 2005).

4.2.2 Intracellular sorting

Although the refractory nature of most cell types to prion infection *in vitro* is a well known phenomenon in prion research, the literature contains little evidence of rigorous investigation of why this might be the case. The decision to investigate the fate of the exogenous PrP^{Sc} revealed an unexpected answer and one that provided a major alternative focus for this thesis.

To examine whether the kinetics of endogenously produced PrP^{Sc} described in the literature for constitutively infected cells are different from the kinetics of exogenous PrP^{Sc} when cells are acutely exposed to prion disease brain material (section 3.8), immunocytochemistry and confocal microscopy were employed. *In vivo* studies

suggest that PrP^{Sc} first accumulates at the cell surface of neurones and dendrites, and either released or internalised from there (Jeffrey *et al.*, 1994b). The intracellular fate of GPI-anchored proteins (GPI-AP) in the endocytic pathway was proposed to be cell type dependent (Fivaz *et al.*, 2002). Differential sorting may be of physiological importance for the intracellular delivery of GPI-AP ligands, as well as perhaps the infectivity of the prion protein. Indeed, certain cell types cannot be infected with prions despite the presence of PrP^{C} (Raeber *et al.*, 1999).

Despite the high level of PrP^{C} expression, the HK cells failed to support PrP^{Sc} replication and to propagate a prion infection in this study (section 3.5). The kinetics of PrP^{Sc} in challenged cells was analysed by dual labelling immunocytochemistry monitored *in situ* by confocal microscopy in attempt to colocalise exogenous PrP^{Sc} with sub-cellular organelles during continuous exposure and particularly during transient exposure in a "pulse-chase" format to a preformed vCJD brain homogenate PrP-6H4 mAb complex. Guanidine pre-treatment was also used to enhance PrP^{Sc} staining and diminish PrP^{C} staining. However, this step did not completely abolish PrP^{C} staining (Taraboulos *et al.*, 1990a; Veith *et al.*, 2009). This problem was circumvented using fluorescence microscopy and image analysis thresholding to discriminate subcellular PrP^{Sc} from PrP^{C}. A similar thresholding approach was recently used by Veith and collaborators (Veith *et al.*, 2009). This approach was necessary because poteinase K digestion, which is generally used to selectively detect PrP^{Sc} interfered with the labelling and integrity of organelles.

The observations indicate that subcellular trafficking of exogenous PrP^{Sc} into perinuclear regions (sections 3.3, 3.4, 3.5) may influence the chances of establishing a prion infection. This is in agreement with findings in other studies showing kinetics

of internalised rPrP or exogenous PrP^{Sc} to be directed to perinuclear region of cell cytoplasm (Caughey and Raymond, 1991; Arnold *et al.*, 1995; Kristiansen *et al.*, 2005; Sanghera *et al.*, 2008).

The HK cells also showed trafficking of exogenous PrP^{Sc} to recycling endosomes and late endosomal/lysosomal structures. The data appears to exclude roles for the Golgi complex and the endoplasmic reticulum in exogenous PrP^{Sc} trafficking. Some positive colocalisation of exogenous PrP^{Sc} with early endosomes was observed at the 24 hours time point of continuous exposure and the 3 to 48 hours time point of the "pulse-chase" study. However, the involvement of early endosomes in exogenous PrP^{Sc} trafficking was not conclusively shown for the time points examined. Whether this colocalisation was real or random was assessed by a quantitative image analysis study in this thesis (section 2.14 and 3.8.3.19). Unfortunately, the pixel-based method used was concluded to be uninformative to compare preferential colocalisation of PrP^{Sc} with cellular organelles, however a particle-based method (which is more appropriate for the analysis of complex morphological structures) confirmed that PrP^{Sc} preferentially colocalises with lysosomes, but not early endosomes.

This phenomenon could be observed both in the continuous exposure and in the "pulse-chase" studies. The endosomal recycling compartment and late endosomes/lysosomes were identified as the likely sites of prion intracellular trafficking in the HK cells. The exogenous PrP^{Sc} was observed in structures positively immunolabeled with the rab11A antibody, resembling recycling endosomes and the late endosomal/lysosomal compartment after 24 hours trafficking through the cell. PrP^{Sc} co-localisation with the LAMP1 and LAMP2b (lysosomal

associated membrane proteins) antibodies gave closely similar patterns in cells continuously exposed to brain homogenate and in "pulse-chase" studies, indicating that the vCJD PrP-6H4 mAb complex did not change or delay the trafficking of exogenous PrP^{Sc} within the cell. The time-course co-localisation data obtained in this part of the thesis is consistent with the following model for PrP^{Sc} trafficking in HK cells (Figure 4.1).

4.2.3 The significance of lysosomal localisation

Recycling endosomes, late endosomes and lysosomes are oxidising environments (Austin *et al.*, 2005) and have been implicated in prion pathogenesis, providing a low pH environment that may favour the partial unfolding of PrP^{C} and allowing more efficient conversion into PrP^{Sc} (Laszlo *et al.*, 1992; Marijanovic *et al.*, 2009; Veith *et al.*, 2009). Exposure of naïve PrP^{C} expressing cells to PrP^{Sc} was shown to induce a rapid lysosomal reactive oxygen species (ROS) response parallel with the internalisation and accumulation of the infectious inoculum in acidic vesicles (Haigh *et al.*, 2011). The positive colocalisation of exogenous PrP^{Sc} with late endosome/lysosomal structures demonstrated in this thesis is in agreement with previous observation of PrP^{Sc} sub-cellular localisation in prion infected cells, or PrP^{Sc} classical intracellular targeting during trafficking (Taraboulos *et al.*, 1990b; McKinley *et al.*, 1991; Caughey *et al.*, 1991; Laszlo *et al.*, 1992; Mayer *et al.*, 1994; Arnold *et al.*, 1995; Harris *et al.*, 1996; Peters *et al.*, 2003; Pimpinelli *et al.*, 2005; Caughey *et al.*, 2009; Veith *et al.*, 2009). Late endosomes, also known as multivesicular bodies (MVBs), are mainly spherical, lacking tubules, and containing many close-packed luminal vesicles (Mellman, 1996; Piper and Luzio, 2001; Russell

et al., 2006). Late endosomes or MVBs fuse directly with lysosomes (Futter *et al.*, 1996; Bright *et al.*, 1997; Mullock *et al.*, 1998; Luzio *et al.*, 2007) and this occurs in the juxtanuclear region of the cell as late endosomes and lysosomes are concentrated near the microtubule-organising centre (Luzio *et al.*, 2007).

The findings in this thesis indicate that uptake of material into the cell is a "complex uptake" and different materials can be taken up along the same endocytic pathway. This was observed in the study employing dual immunolabelling for PrP^{Sc} and glial fibrillary acidic protein (GFAP) (section 3.5). Both PrP^{Sc} and GFAP proteins accumulated in the cytosol and were uniformly distributed at the same intracellular site of the cell, suggesting a general rather than a specific uptake mechanism.

Late endosomes are the last sorting station in the endocytic pathway before lysosomes (Sobo *et al.*, 2007). The trafficking of late endosome-like vesicles containing PrP^{Sc} to lysosomes was proposed to play a crucial role in the accumulation or elimination of PrP^{Sc} (Okemoto-Nakamura *et al.*, 2008). Lysosomes are dynamic organelles that accept and degrade macromolecules from endocytic, autophagic, phagocytic and secretory membrane-trafficking pathways (Luzio *et al.*, 2007). The observation of the exogenous PrP^{Sc} clearance from HK and hESC in the recovery assay (section 3.6) and the documented trafficking of PrP^{Sc} into lysosomal vesicles (section 3.8) are consistent with the previous studies implicating lysosomal proteases in PrP^{Sc} degradation (Beringue *et al.*, 2000; Supattapone *et al.*, 2001; Luhr *et al.*, 2002; Dormont, 2003; Jeffrey *et al.*, 2003; Kiachopoulos *et al.*, 2004; Mallucci and Collinge, 2005; Rybner-Barnier *et al.*, 2006; Dron *et al.*, 2009; Sassa *et al.*, 2010).

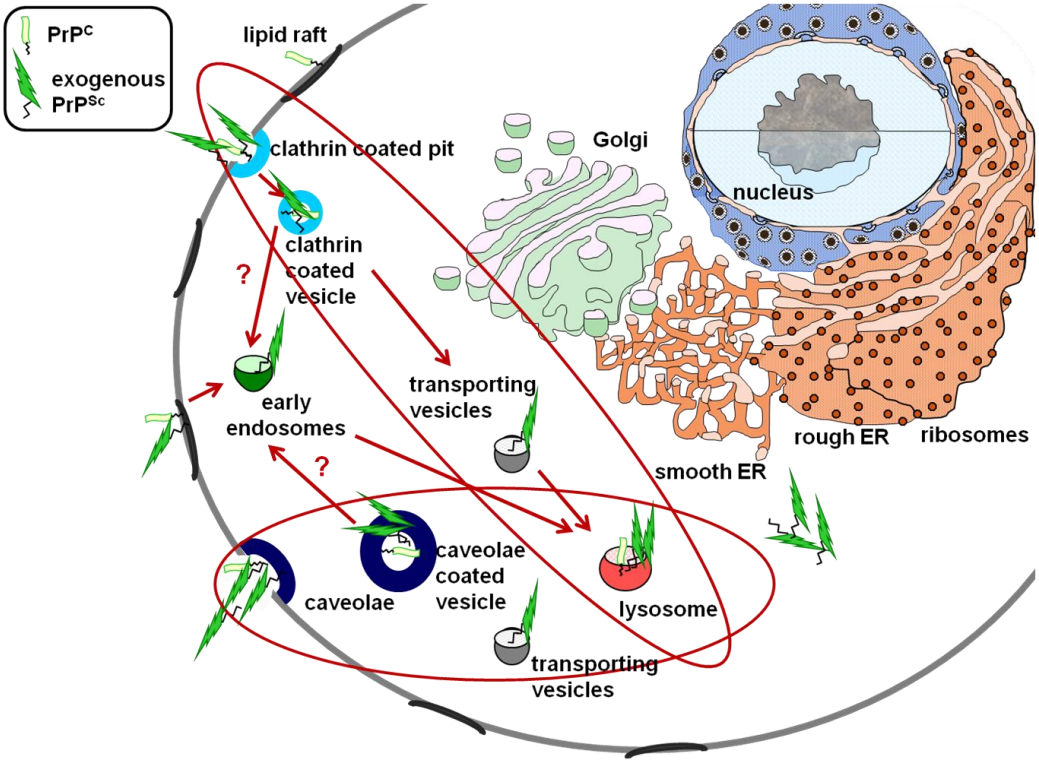


Figure 4.1: Trafficking of exogenous PrP^{Sc} within the cell
In summary, the exogenous PrP^{Sc} is predominately taken up by caveolae-coated vesicles preceding the uptake by clathrin-coated pits. Trafficking of exogenous PrP^{Sc} in early endosomes, Golgi complex and endoplasmic reticulum was shown to be negative in HK cells. Trafficking of late endosome vesicles containing PrP^{Sc} to lysosomes was proposed to be the path the exogenous PrP^{Sc} undertake in the FDC-like HK cell.

4.2.4 PrP^{Sc} clearance

The possibility that certain cells have the ability to degrade PrP^{Sc} has recently come into focus. Prions are thought to be resistant to proteolytic degradation. However, evidence for relatively efficient natural clearance mechanisms has been reported (Mallucci and Collinge, 2005). $PrP^{0/0}$ mice that are unable to replicate prion infectivity (Bueler *et al.*, 1993) were able to reduce intracerebrally introduced lethal dose of prion inoculum (for wild type mice) below detection levels by a natural mechanism within two weeks (Mallucci and Collinge, 2005). Moreover, scrapie infected N2a cells are able to clear PrP^{Sc} in three days after transient treatment with

monoclonal antibodies against PrP. Clearance was suggested to be the result of the prevention of formation of new PrP^{Sc} in combination with intracellular degradation of existing PrP^{Sc} (Peretz *et al.*, 2001; Enari *et al.*, 2001).

Spleen and lymph nodes of the lymphoreticular system seem to be critical for PrP^{Sc} replication and neuroinvasion in some murine scrapie models (Blattler *et al.*, 1997; Mabbott *et al.*, 2000). Macrophages were implicated as alternative sites of prion accumulation and replication in the absence of functional FDCs. However, they were also demonstrated to be able to clear both inoculum and newly formed PrP^{Sc} *in vitro* and *in vivo* (Carp and Callahan, 1981; Beringue *et al.*, 1999; Beringue *et al.*, 2000).

A recent study demonstrated that bovine phagocytic cells have strong degradation abilities against exogenous PrP^{Sc} *in vitro* (Sassa *et al.*, 2010). Additional to murine macrophages (Carp and Callahan, 1981), mouse and rat dendritic cells (DCs) have also been shown to degrade TSE agents (Luhr *et al.*, 2002; Huang *et al.*, 2002; Rybner-Barnier *et al.*, 2006). Murine bovine marrow-derived DCs were shown to degrade PrP^{Sc} *in vitro*, but are considered to still have the capacity to spread prion infectivity (Luhr *et al.*, 2002). Phagocytic cells, such as macrophages, appear to degrade PrP^{Sc} (Sassa *et al.*, 2010), but dendritic cells may also have the potential to deliver the prion agents to FDCs where early accumulation of PrP^{Sc} occurs (Huang *et al.*, 2002; revieved by Mabbot and MacPherson, 2006; revieved by Heikenwalder *et al.*, 2007; reviewed by Cobb and Surewicz, 2009). A similar dual function is thought to occur in the processing of other pathogens. For example, CSF-1 dependent cells, which include population of macrophages and DCs, can protect against systemic infection with *Listeria monocytogenes*, but they have also been shown to facilitate its neuroinvasion (Jin *et al.*, 2002).

4.3 RELEVANCE TO PRION DISEASE AETIOLOGY, PATHOGENESIS AND RISK

4.3.1 Sporadic and familial forms

Internalisation of misfolded or aggregated PrP and its subsequent degradation in a lysosomal compartment might function as a cellular mechanism to eliminate non-native, presumably non-functional PrP conformers endocytosed into the cells. This process might play an important role in the balance between clearance and propagation of prions in an infected host, but it may also play a role in avoiding sporadic or spontaneous forms of human prion disease and in delaying familial forms in individual that carry mutations in *PRNP*. Although the evidence is limited, one could hypothesize that small amounts of PrP^{Sc} may be produced in cells throughout life which are degraded efficiently by the lysosomal machinery. The so called "silent prions" described by Yuan *et al.,* (2006) might represent such a molecular species. However, when the amount of the protein destine for degradation overloads the system (perhaps due to invasive PrP^{Sc} replication) or when the degradative machinery begins to fail (due to aging, insult or genetics) the endogenous PrP^{Sc} may reach a threshold for developing a self-sustaining pathogenic prion disease. Interestingly, HK cells appear to be able to respond to their environment by increasing the numbers of LAMP1- and LAMP2b-positive lysosomes. Whether such a mechanism occurs *in vivo* in response to prion propagation is not known.

The molecular mechanisms involved in neurodegeneration in prion diseases are incompletely understood, but are thought to involve multiple processes operating simultaneously and synergistically in the brain, including spongiform degeneration, synaptic alterations, dendritic atrophy, vacuolisation, autophagy, microglial activation, oxidative stress, brain inflammation, neuronal death and the accumulation

of protein aggregates. The notion that neurodegeneration is caused by the formation and accumulation of PrP^{Sc} is most likely an oversimplification. The formation and accumulation of PrP^{Sc} in combination with an inflammatory glial response may have a synergistic deleterious effect on neurones producing a fatal threshold level of cellular stress and resulting in neuronal apoptosis. It may be desirable to co-culture neuronal cells and glial cells infected with prions to help elucidate the mechanism of prion-induced neurodegeneration.

4.3.2 Acquired forms

Acquired human prion diseases occur in the form of vCJD (orally acquired human BSE), kuru (orally acquired presumed sCJD) and iatrogenic CJD (acquired through a variety of medical routes). It is generally accepted that exposure of the UK population to BSE through food was widespread and extensive, but this is not reflected in the current incidence of vCJD (Valleron *et al.*, 2001; Ferguson *et al.*, 2002; Ghani *et al.*, 2003). A poorly recognised but general ability of human cells to take up and degrade PrP^{Sc} may be one component part of an explanation for this discrepancy, in addition to better recognised potential explanations, such as a substantial species barrier existing between cattle and humans (Bishop *et al.*, 2006).

Recent evidence of asymptomatic infected carriers in the UK population, as well as the four cases of transfusion-related secondary vCJD cases suggest a possible "underground propagation" of PrP^{Sc} within the human population (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006; Bishop *et al.*, 2006; Hewitt *et al.*, 2006; HPA, 2007; Peden *et al.*, 2010) (http://www.cjd.ed.ac.uk/TMER/TMER.htm). All definite clinical cases of vCJD have the MM rather that MV or VV configuration at

the position 129 of the *PRNP* gene (Zeidler *et al.*, 1997; Mead *et al.*, 2007; Mead *et al.*, 2009; Kaski et al., 2011) (http://www.cjd.ed.ac.uk). To date, only one possible case of vCJD with an MV genotype has been reported, althoughr the post-mortem examination to confirm the diagnosis was not performed (Green *et al.*, 2010; Heath *et al.*, 2010). Nevertheless, there have been two reported MV recipients of a vCJD implicated blood transfusion and blood products which, despite being clinically unaffected, shown signs of secondary vCJD infection, specifically accumulation of the prion disease-associated protein in their spleen at the autopsy examination (Peden *et al.*, 2004; Peden *et al.*, 2010). Transmission studies of vCJD to the three lines of humanised transgenic mice indicated that the MM and MV genotypes might be equally susceptible to vCJD (but with different incubation periods) and that VV individuals may also be susceptible (including very long incubation times and a lengthy subclinical phase) (Bishop *et al.*, 2006). The inability to establish a constitutive infection in the FDC-like HK cells (VV) *in vitro* in this thesis could be seen to be consistent with this interpretation.

Despite the rarity of the prion diseases, the emergence of variant CJD, linked to the consumption of cattle infected with BSE, and more recently transmission-associated secondary transmission, has brought TSEs to the forefront of human health concerns (Will *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997a; Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006). Another potential route for accidental person-to-person transmission of the infection, beside surgery, blood transfusion and tissue transplantation, are future stem cell therapies. As most current culture systems supporting human embryonic stem cell (hESC) self-renewal and differentiation rely on both direct and indirect exposure to animal or human cells, and on the use of cell

products such as bovine serum or human purified proteins, (De Sousa *et al.*, 2006) it is possible that hESC could represent a potential risk of prion transmission.

The study carried out in this thesis was the first to explore the response of hESC after acute prion exposure and evaluate the observations within the context of inadvertent prion transmission by future clinical application of hESC or their differentiated derivates and the consequences for public health (section 3.6, 3.7) (Krejciova *et al.*, 2011). The observations are provocative. Although hESC take up and rapidly clear PrP^{Sc}, the potential long-term effect of such exposure is not known and further investigation is required. The observation that exposure of hESC to prion diseased brain material does not result in gross noticeable cytotoxicity was an interesting finding in itself. This may be a further confirmation of the non-toxic nature of PrP^{Sc} when acquired by cells and circumscribed by its sub-cellular localisation. However, whether this could result from the nature of the hESC (undifferentiated, i.e. non-neuronal phenotype) merits further investigation. These experiments were short-term, did not address infectivity directly and did not involve hESC differentiated to relevant phenotypes, such as neurons. Whether all exposed cells are able to clear PrP^{Sc} entirely, and whether they do so before a prion infection may be established remains, to be explored, as does the consequence of cellular differentiation of hESC cells previously exposed to infectious prions. This is important because clinical applications of hESC will involve cells that have been subjected to differentiation *in vitro* prior their use in stem cells therapies or transplantation. Differentiation-associated change in PrP^{C} expression may cause hESC to become susceptible to prion infection from media constituents or may permit a low level of contamination to become an established infection.

The possibility that future therapies based on hESC technology might present a risk of prion transmission cannot be discounted out of hand. Despite the low likelihood of animal and human prion contamination of hESC, the consequences of any such event could be serious, especially when one considers the number of patients that might receive cellular therapies from one individual cell line. These concerns coupled with our limited knowledge of the potential risks posed by known and emergent human and animal TSEs world-wide suggest that further investigation of the potential of hESC to harbour and propagate prions are merited. They also provide a rationale for the development of methods of hESC derivation and cultivation that minimise exposure to possible sources of human and animal prions.

5. LIST OF REFERENCES

5.1 REFERENCES

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5.2 LIST OF BOOKMARKS OF ONLINE RESOURCES

http://www.cjd.ed.ac.uk/vcjdworld.htm

http://www.fbs.leeds.ac.uk/staff/Hooper_N/prion.htm

http://biogps.org/#goto=genereport&id=5621

http://www.cmpharm.ucsf.edu/cohen/

http://vla.defra.gov.uk/science/docs/sci_tse_stats_gen.pdf

http://www.cjd.ed.ac.uk/TMER/TMER.htm

http://www.docs.csg.ed.ac.uk/Safety/bio/guidance/transport/summary_catB.pdf

http://vla.defra.gov.uk/services/ser_tse_archive.htm

http://www.cjd.ed.ac.uk

http://www.docs.csg.ed.ac.uk/Safety/bio/guidance/transport/summary_catB.pdf

http://www.dh.gov.uk/ab/ACDP/index.htm

6. APPENDIX 1

Some data from this thesis were published in The Journal of Pathology and reprint of the article is enclosed in this appendix 1 (358-368). Full permission has been granted form the publishing journal and this is enclosed on the page 369.

Krejciova, Z., Pells, S., Cancellotti, E., Freile, P., Bishop, M., Samuel, K., Barclay, G. R., Ironside, J. W., Manson, J. C., Turner, M. L., De Sousa, P., and Head, M. W. (2011) Human embryonic stem cells rapidly take up and then clear exogenous human and animal prions *in vitro*. *The Journal of Pathology* **223,** 635-645.

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ORIGINAL PAPER

Human embryonic stem cells rapidly take up and then clear exogenous human and animal prions *in vitro*

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Abstract

Susceptibility to prion infection involves interplay between the prion strain and host genetics, but expression of the host-encoded cellular prion protein is a known prerequisite. Here we consider human embryonic stem cell (hESC) susceptibility by characterizing the genetics and expression of the normal cellular prion protein and by examining their response to acute prion exposure. Seven hESC lines were tested for their prion protein gene codon 129 genotype and this was found to broadly reflect that of the normal population. hESCs expressed prion protein mRNA, but only low levels of prion protein accumulated in self-renewing populations. Following undirected differentiation, up-regulation of prion protein expression occurred in each of the major embryonic lineages. Self-renewing populations of hESCs were challenged with infectious human and animal prions. The exposed cells rapidly and extensively took up this material, but when the infectious source was removed the level and extent of intracellular disease-associated prion protein fell rapidly. In the absence of a sufficiently sensitive test for prions to screen therapeutic cells, and given the continued use of poorly characterized human and animal bioproducts during hESC derivation and cultivation, the finding that hESCs rapidly take up and process abnormal prion protein is provocative and merits further investigation.

Keywords: prions; stem cells; Creutzfeldt–Jakob disease (CJD); BSE; prion protein (PrP); cell culture; iatrogenic transmission

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Introduction

Human stem cell therapy offers great promise for a wide variety of currently intractable conditions, but safety, in addition to efficacy, is a key requirement for clinical application. Ensuring the pathogen-free status of therapeutic cells can be relatively easily accomplished for conventional pathogens with nucleic acid genomes such as bacteria and viruses. However, the potential for inadvertent prion transmission by emerging cellular technologies has received comparatively little attention [1–3]. These pathogens differ from bacteria and viruses in that they are not known to contain a defined nucleic acid genome, but instead are associated with (and may be solely composed of) an abnormally folded form of the host-encoded prion protein [4]. In part because of their unique biology [5–7], prions can be difficult to detect, to inactivate and to remove [8–10].

Zoonotic transmission of bovine spongiform encephalopathy (BSE) is the acknowledged cause of variant Creutzfeldt–Jakob disease (variant CJD) in humans [11–13] and variant CJD has recently been shown to be transmissible by blood transfusion [14–17]. The more prevalent sporadic form of Creutzfeldt–Jakob disease has a long history of inadvertent person-to-person transmission during medical procedures, such as human growth hormone therapy, dura mater grafting and corneal transplantation, resulting in iatrogenic CJD [18,19]. Given this precedent, it appears prudent to consider the potential risks of prion transmission in advance when new therapies or treatments are being developed, especially ones in which materials of human or animal origin are transfused or transplanted.

Most current culture systems supporting human embryonic stem cell (hESC) self-renewal and differentiation rely on both direct and indirect exposure to animal or human cells, and on the use of cell or animal products such as serum or purified proteins [20]. These

components represent a potential risk of infection, as might the cells themselves. Prion diseases present a particular problem in this context because the diseases are transmissible by a variety of routes and once acquired can have a prolonged clinically silent phase followed by a rapid irreversible clinical phase [21]. Increased surveillance and research performed largely as a consequence of the identification of BSE and variant CJD in the UK has led to the identification of previously unrecognized forms of both animal and human prion disease in countries worldwide [22,23]. Whether any of these animal or human diseases might have zoonotic or iatrogenic potential remains to be seen.

According to the prion hypothesis, the abnormal form of the prion protein, termed PrP^{Sc}, constitutes a novel pathogen (or prion) necessary for disease transmission and pathogenesis, exerting its effect through the autocatalytic post-translational conversion of the host-encoded cellular prion protein, PrP^{C} [4]. Although the exact relationship between PrP^{Sc}, infectivity and disease is controversial [24,25], PrP^{Sc} remains the principal diagnostic marker for prion diseases [21]. Consistent with the prion hypothesis, PrP^{C} expression is a prerequisite for susceptibility [26,27]. In human prion diseases the phenotype, susceptibility and incubation period are all governed, in part, by host genetics. In particular, the methionine/valine (M/V) polymorphism at codon 129 of the prion protein gene (*PRNP*) has a powerful effect [28–31].

Materials and methods

hESC lines

hESC lines RH1, RH3, RH4, RH5, RH6 and RH7 and RCM-1 were used in this study and have been described previously [32,33]. They were derived under an HFEA license (No. R0136) permitting the use of donated embryos in research, the creation of embryos and the derivation of human embryonic stem cells. Full informed consent for research use was obtained in advance from the donor couple.

PRNP codon 129 genotyping and *PRNP* expression studies

hESCs had their *PRNP* codon 129 genotype determined as described previously [28]. The primers and reaction conditions used for the RT–PCR experiments were also as previously described [34]. Detection of PrP^{C} by immunofluorescence employed the monoclonal antibody SAF32 (SPIbio; Cambridge Biosciences, UK) and nuclear counterstaining with DAPI (Invitrogen, UK).

Routine cultivation of hESCs

hESCs were cultured in feeder-free conditions, in human dermal fibroblast (Cascade Biologics, UK)-conditioned medium supplemented with 4 ng/ml bFGF (HDF^{-} CM^{+}) on Matrigel [35], with modifications as described previously [33]. The hESC passage numbers used in the experiments varied between 45 and 65 (RCM-1), and 60 and 85 (RH1), with the exception of those cultures used for experiments involving flow cytometry, which were conducted on passage number 50 of both the RCM-1 and RH1 cell lines.

In vitro differentiation of hESCs

RCM-1 and RH1 hESC cultures were disaggregated to a single cell suspension, using 0.025% trypsin–0.01% EDTA (TED) in PBS, and plated out into AggreWell™ 400 plates (Stem Cell Technologies, France) at a density of 2000 cells/microwell in the presence of 10 μM Y-27632 ROCK inhibitor. After culture for 24 h at 37 °C, 5% CO_2/air embryoid bodies had formed in each microwell. Embryoid bodies were harvested and cultured for 7 days in 15% knockout serum replacement (Invitrogen) in DMEM with L-glx and non-essential amino acids in Corning ultra-low adherent six-well plates (ie suspension culture). After 7 days in suspension culture, the embryoid bodies were transferred to Matrigel-coated wells and cultured for up to 21 days in the same medium, supplemented with 10^{-6} M retinoic acid and then harvested for flow cytometry.

Flow cytometry

RCM-1 and RH1 hESCs were harvested for flow cytometry by washing in PBS and generating a single cell suspension with TED, resuspended in FACS-PBS (PBS/0.1% BSA/0.1% NaN_3) and then split into aliquots for staining (all antibodies were optimized by titration before use). Primary antibodies directed against PrP (6H4 and 8H4): Tra 1–60 (ES cell marker; BioLegend, UK), CXCR4, which is also known as CD184 (endodermal marker; BD Pharmingen™, UK); CD56 (ectodermal marker; eBioscience) and CD140 (mesodermal marker; BD Pharmingen™, UK), were added and the cells incubated at 4 °C for around 30 min. The cells were washed with PBS and, for unconjugated primary antibodies (6H4, 8H4), secondary antibody was added. The secondary antibodies used were FITC–goat anti-mouse IgG (Fcγ subclass 1-specific; Jackson ImmunoResearch Laboratories, used with 8H4). The cells were incubated for 30 min at 4 °C, and washed with PBS prior to analysis with a Becton-Dickinson FACSCalibur flow cytometer equipped with 488 and 633 nm lasers. Data for up to 50000 events were acquired and analysed using CellQuest and FCS Express software, respectively.

Human and animal tissue specimens

Brain tissue from well-characterized UK cases of definite sporadic and variant CJD and Alzheimer's disease, with consent for research use, was obtained from the National CJD Surveillance Unit Brain Bank (LREC 2000/4/157). The variant CJD and Alzheimer's

disease brain tissue were from single cases, both of which were homozygous for methionine at codon 129 of *PRNP* (MM). Three different cases of sporadic CJD were used. Two were of the most commonly occurring subtype, MM1, and the third was of the second most frequently occurring subtype, VV2. The single isolate of BSE-affected cattle brain stem was supplied by the Veterinary Laboratory Agency TSE Archive (Weybridge, UK). Detection of PrP^{Sc} in brain homogenates and culture media was confirmed by proteinase K digestion and western blot analysis as described previously [36].

PrP^{Sc} uptake by hESCs

hESCs were plated onto Matrigel (in preliminary experiments) or 95% hyaluronan/5% matrigel-coated (to minimize background staining) four-well glass chamber slides at a density of 10^4–10^5 cells/cm^2. The next day they were treated with a cleared, sonicated 10% brain homogenate prepared by homogenization in PBS/5% glucose buffer. Routinely, cells were exposed continuously to brain homogenate at a final w/v concentration of 1% in cell culture medium for periods of up to 48 h, followed by immunocytochemistry for prion protein and other markers, and analysed by confocal microscopy.

Immunofluorescence and confocal microscopy

Samples for fixed and permeabilized cell staining were washed with PBS, fixed in chamber slides with 4% paraformaldehyde (PFA) in PBS, permeabilized with 0.1% Triton X-100 in PBS, blocked with 3% bovine serum albumin (BSA) in Dulbecco's modified PBS, and incubated with the PrP primary antibodies in blocking solution. The mAb 8H4 was used at a 1 : 800 dilution overnight, whereas the 6H4 (Prionics, Switzerland) and 3F4 (Dako, UK) mAbs were used at dilutions of 1 : 1000 and 1 : 200, respectively, for 30 min at 37 °C and then 30 min at room temperature with shaking. Alexa 488-conjugated secondary antibody (at a 1 : 200 dilution; Invitrogen) was used for 60–90 min, followed by counterstaining with DAPI (at a 1 : 17 000 dilution) for 15 min. Samples for live cell staining were washed with PBS, blocked with 3% BSA in Dulbecco's PBS and incubated with the primary antibody 8H4 (at a 1 : 800 dilution) for 90 min in blocking solution. The Alexa 488-conjugated secondary antibody was used as described above, followed by counterstaining with DAPI (at a 1 : 17 000 dilution) for 15 min. Samples for glial fibrillary acidic protein (GFAP) immunostaining were fixed and permeabilized as for PrP staining, but a primary anti-GFAP antibody (at a 1 : 600 dilution; Dako) was incubated with the cells for 30 min, followed by the Alexa 546-conjugated secondary antibody (at a 1 : 200 dilution; Invitrogen) for 60 min, and DAPI (at a 1 : 17 000 dilution) for 15 min. The slides were mounted and imaged with an LSM5 Pascal laser scanning confocal microscope (Zeiss).

Table 1. Comparison of *PRNP* codon 129 frequencies in human embryonic stem cell lines and the normal healthy population

Codon 129	Normal population (%)	hESC [*n* (%)]	Identity[a]
MM	44	3/7 (43)	RH3, RH4, RCM-1
MV	45	3/7 (43)	RH1, RH5, RH7[b]
VV	11	1/7 (14)	RH6

[a]As described previously [32,33]. [b]Showing a (non-pathogenic) 24 bp deletion of the 129-M allele.

Semi-quantitative assessment of photomicrographs

To give a measure of the change in PrP immuncytochemical signal over time, we used the histogram function in Adobe Photoshop® to separately quantify the blue and green fluorescence in the photomicrographs shown. The green fluorescence pixel value (representing the PrP immunostaining signal) was divided by the blue fluorescence pixel value (representing DAPI stained nuclei), thus normalizing PrP immunostaining for cell number. These values were plotted as arbitrary fluorescence units, using Microsoft office Excel®.

Results

We tested seven independent hESC lines for their *PRNP* codon 129 polymorphism (Table 1). The data are consistent with the frequencies of the *PRNP* codon 129 polymorphism in the general healthy population [28], suggesting that *PRNP* codon 129 polymorphic variation does not affect hESC viability.

We analysed the hESC lines, grown in feeder-free conditions, by RT–PCR for expression of the *PRNP* gene. Human dermal fibroblasts (HDFs) employed as feeder cells during hESC isolation and for the conditioning of culture media were also evaluated. The HDFs and the six hESC lines tested expressed *PrP* mRNA (Figure 1A). This result was consistent with Affymetrix U133plus2 genechip data for three of the lines (RCM-1, RH-1 and RH-3) showing detectable *PRNP* expression (data not shown). Because the majority of the healthy Caucasian human population are heterozygous at codon 129 of the prion protein gene, we then focused our attention on the RH1 hES cell line. Immunocytochemistry indicated low levels of PrP^C in or on RH1 hESCs (Figure 1Bd). Flow cytometry dual-staining of RH1 cells for the hESC 'stemness' marker Tra 1–60 and PrP^C indicated that a significant minority (10–15%) of hESCs (ie Tra 1-60-positive cells) were PrP^C-positive (Figure 1C). Similarly, only a subpopulation of presumably early differentiating (Tra 1-60-negative) cells were PrP^C positive. Analysis of the RCM-1 hESC line (*PRNP* codon 129-MM) by flow cytometry for Tra 1–60 and PrP^C gave similar results (data not shown).

To determine whether changes in PrP expression were associated with cell lineage specification, we subjected RH1 hESCs to an undirected *in vitro* differentiation protocol and sampled the cells after 1, 2 or

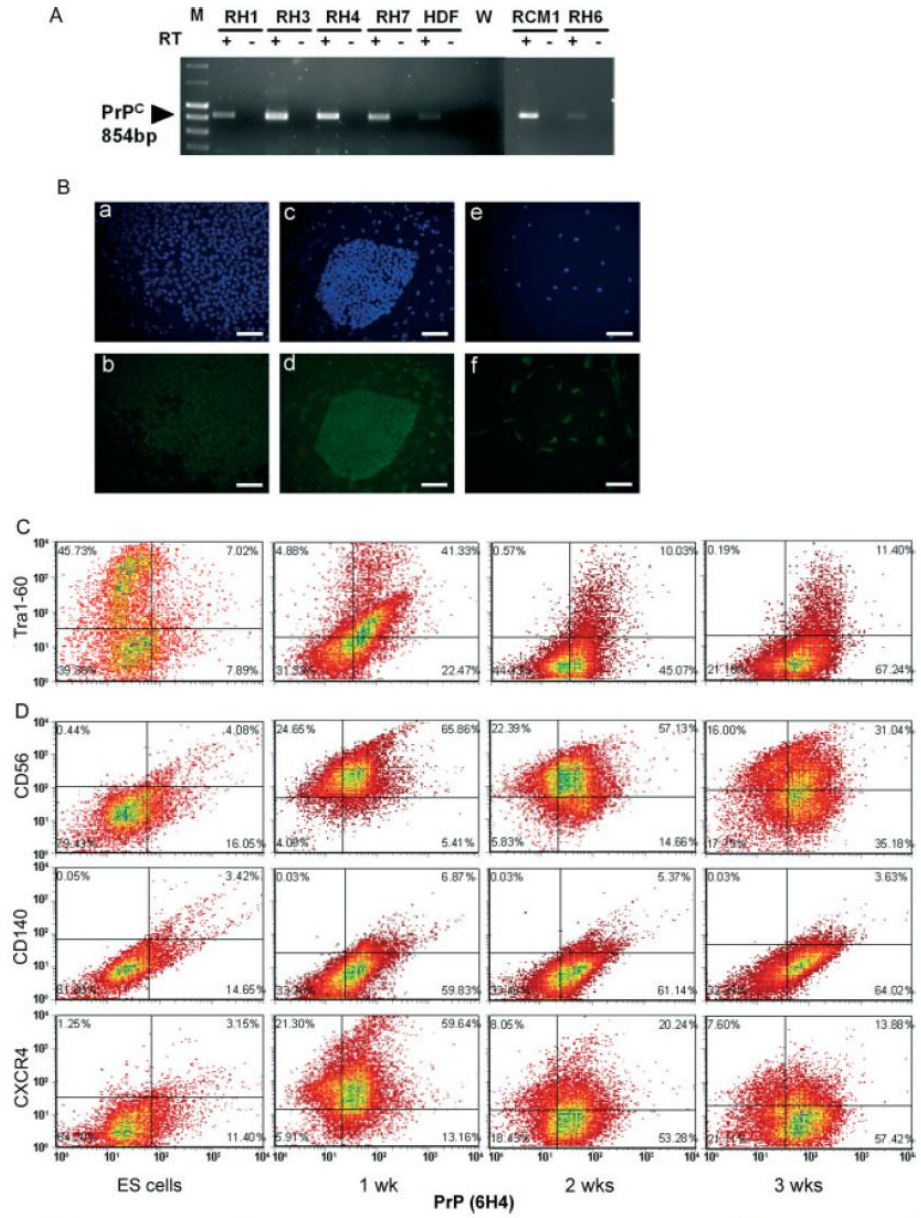

Figure 1. (A) hESCs and human dermal fibroblasts express the human *PRNP* gene, as shown by RT–PCR. M, molecular weight marker; RT+ or −, presence or absence of reverse transcriptase in reverse transcription reaction; W, water; RH*x* and RCM-1, various hESC lines; HDF, human dermal fibroblasts. (B) Immunofluorescence staining of RH1 hESCs with the SAF-32 PrP monoclonal antibody shows visible staining on the surface of stem cell clusters in normal self-renewing cultures: (a) DAPI-stained nuclei of hESCs; (b) the same field with no SAF-32 antibody, shown as a negative control; (c) DAPI-stained nuclei of hESCs in a dense clump; (d) the same field with the SAF-32 PrP antibody; (e) DAPI-stained nuclei of HDFs; (f) SAF-32 staining of the same field. Scale bar = 100 μm. (C) Flow cytometry (RH1 hESC line) using two-colour staining for the stemness marker Tra 1–60 (*y* axis) and PrP^C (*x* axis) of cells grown under self-renewing conditions (ES cells) or after 1 week (1 wk), two weeks (2 wks) or three weeks (3 wks) of differentiation. (D) Flow cytometry (RH1 hESC line), using two-colour staining for PrP^C and cell surface markers of various lineages (CD56, ectoderm; CD140, mesoderm; CXCR4, endoderm) when cells were grown under self-renewing conditions (ES cells) or during differentiation.

3 weeks for analysis by flow cytometry, co-staining for PrP^C and surface markers characterizing endodermal, mesodermal and ectodermal lineages, in addition to co-staining for Tra 1–60. We observed that PrP^C expression rose rapidly during differentiation, concurrent with a decrease in Tra 1-60-positive cells (Figure 1C). Both PrP levels and the percentage of PrP^C-positive cells increased until the great majority of cells were PrP^C-positive. By 7 days of differentiation the majority of cells expressed significant levels of PrP^C, whatever their co-expressed lineage marker (Figure 1D). Even after 3 weeks in differentiation conditions, there remained a small population of Tra 1-60-positive cells, and these were almost all PrPC-positive. Similar results were obtained following *in vitro* differentiation of RCM-1 cells (data not shown).

Next we exposed hESCs to crude extracts of CJD, BSE and Alzheimer's disease-affected brain, the latter serving as a non-prion disease control. Because variant and sporadic CJD disproportionately affects those homozygous for methionine at codon 129 of the prion protein gene, we initially focused on the RCM-1 hES cell line (*PRNP* codon 129-MM). Exposure to brain homogenate did not result in gross changes in cell morphology, growth characteristics or viability as judged by regular observation of the cultures using differential interference contrast microscopy and comparison with parallel unexposed control cultures (data not shown). We used immunocytochemistry and confocal microscopy of fixed and permeabilized cells to monitor the fate of PrP^{Sc} added to the cultures. Unchallenged hESCs (Figure 2D, H, L, P, T) and those challenged with brain material from non-CJD patients (Figure 2C, G, K, O, S) showed little or no detectable PrP under the immunostaining conditions used in these particular experiments. However, RCM-1 cells challenged with variant CJD and BSE brain showed intensely staining accumulations of PrP, increasing with exposure time, suggesting uptake of PrP^{Sc} from the medium (Figure 2A, E, I and B, F, J).

Although fixed and permeabilized RCM-1 cells showed strong PrP immunostaining, live cell staining resulted in no signal (Figure 2M–P), consistent with the positive immunostaining being intracellular rather than resulting from the brain material deposited or adsorbed on the external surface of the cells. The results were qualitatively similar when cells of the RH1 line (*PRNP* codon 129-MV) were used (Figure 2Q–T). When viewed at high magnification, the immunostaining for PrP was intense and had a coarse granular appearance in variant CJD-exposed RCM-1 cells (Figure 3Aa), as compared to the fainter patchy PrP immunostaining of unexposed cells (Figure 3Ab). The use of immunohistochemical pretreatments (guanidine and proteinase K), which accentuate PrP^{Sc} staining and diminish PrP^C staining, confirmed that the immunostaining resulted from PrP^{Sc} (Figure 3B, C). The absence of immunostaining in BSE-exposed hESCs with anti-PrP antibody 3F4 (which recognizes human, but not bovine, PrP; Figure 3Bb) further confirmed that the immunostaining with mAb 6H4 depended upon uptake of exogenous bovine PrP^{Sc} from the medium by these cells (Figure 3Ba). The absence of 3F4 immunostaining of hESCs exposed to BSE also implies that under these conditions there had been no detectable conversion of human cellular PrP^C to PrP^{Sc} as a result of BSE exposure. hESC uptake of PrP^{Sc} was not particular to BSE/variant CJD infected brain material. Exposure of the cells to medium containing crude extracts of sporadic CJD brain of either of the most common subtypes (MM1 and VV2) produced similar results (Figure 3Ca, b). Immunostaining for glial filaments in cells exposed to variant CJD and Alzheimer's disease brain indicated that the material taken up by the hESCs is complex, including brain components other than PrP^{Sc} (Figure 3D).

We then used the PrP^{Sc} immunostaining protocol that included guanidine and proteinase K pre-treatment to examine the kinetics of PrP^{Sc} clearance in RCM-1 cells (Figure 4). When the variant CJD and BSE brain-spiked medium was withdrawn after 48 h of exposure (Figure 4A, B) and the cells were allowed to continue growing in control medium, the intensity and extent of PrP^{Sc} immunostaining could be seen to have fallen at 24 h (Figure 4D, E) and to be further reduced after 48 h (Figure 4G, H). By the 72 h time point, only low or background staining characterized the variant CJD and BSE exposed cultures (Figure 4J, K). However, above-background levels of PrP^{Sc} immunostaining remained in some cells after 72 h (Figure 4M, N). Cells exposed to Alzheimer's disease brain homogenate remained negative for PrP^{Sc} immunostaining throughout (Figure 4C, F, I, L, O). Using the DAPI fluorescent signal to normalize for cell number, the general trend in reduction of PrP^{Sc} immunofluorescence over time in the above representative micrographs is clear (Figure 5). Western blot analysis for PrP^{Sc} in the variant CJD and BSE brain-spiked medium before and after incubation with the cells suggests extensive uptake of PrP^{Sc} from the medium by the cells (Figure 6A, B, lanes 1 and 2). Fresh (unspiked) medium then added to the cells for a further 24 h showed only trace levels of PrP^{Sc}, suggesting that the majority of the material taken up remained cell-associated rather than being shed back into the medium during the course of the experiment (Figure 6A, B, lanes 2–5).

Discussion

This study is the first to examine prion biology in human embryonic stem cells and consider the results within the context of potential inadvertent prion transmission through clinical application of hESCs or their differentiated derivatives. Expression of the normal cellular isoform of the prion protein PrP^C is thought to be a prerequisite for susceptibility to prion infection. The physiological role of PrP^C, even

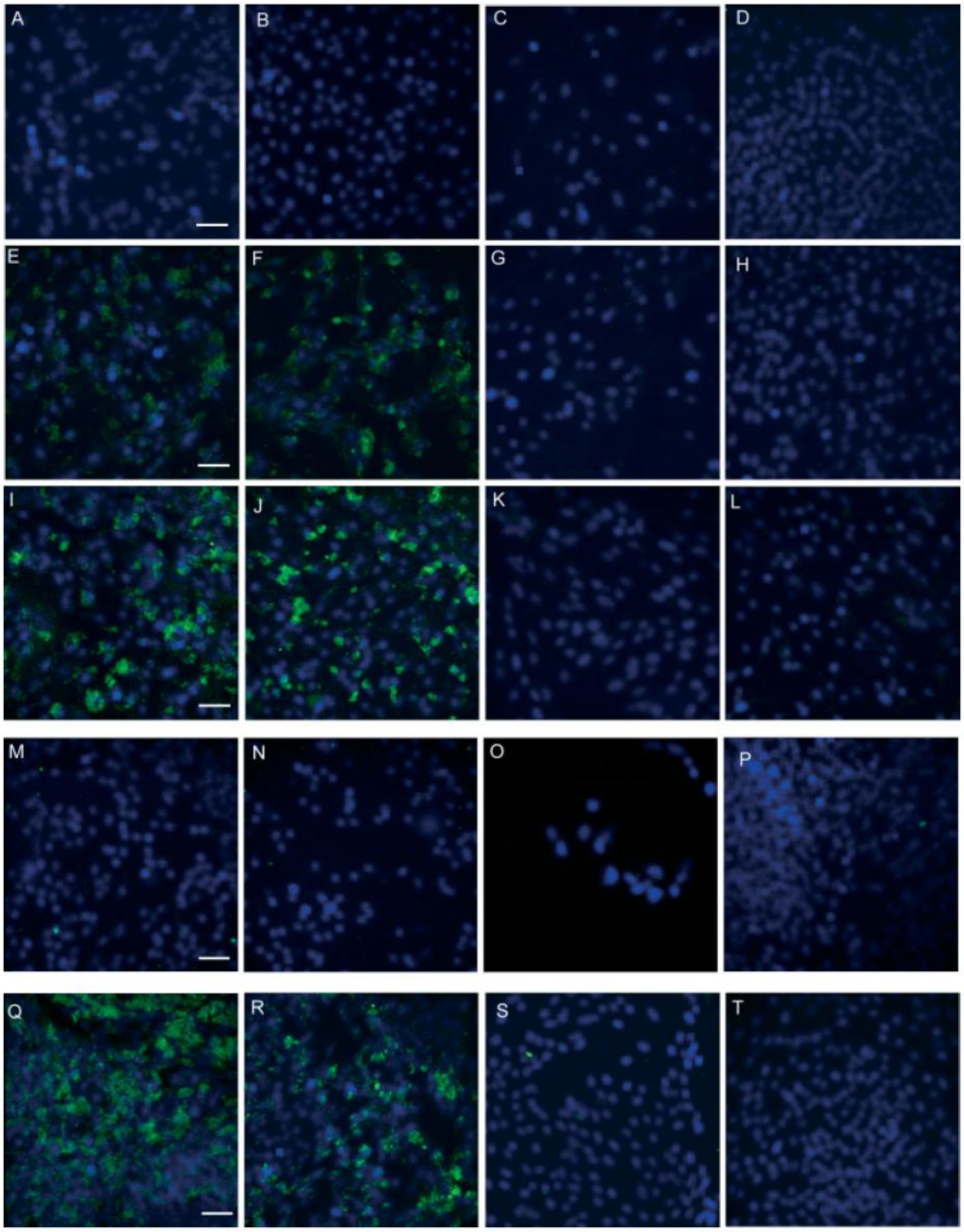

Figure 2. Progressive PrP uptake in hESCs continuously exposed to BSE and variant CJD brain homogenate shown by immunocytochemistry. hESCs of the RCM-1 cell line (*PRNP* codon 129-MM genotype; A–P) and the RH1 cell line (129-MV genotype; Q–T) were exposed to BSE (A, E, I, M, Q), variant CJD (B, F, J, N, R) or non-CJD (Alzheimer's disease) (C, G, K, O, S) 1% brain homogenate, or grown in control medium without brain homogenate (D, H, L, P, T) for 1 h (A–D), 24 h (E–H) or 48 h (I–T). hESC cultures (A–L and Q–T) were then fixed, permeabilized and immunostained for PrP with the antibody 8H4 (green) and the nuclei counterstained with DAPI (blue). Immunostaining without prior fixation and permeabilization is also shown for the RCM-1 cell line (*PRNP* codon 129-MM; M–P). Scale bars (shown in left hand column only) = 50 μm.

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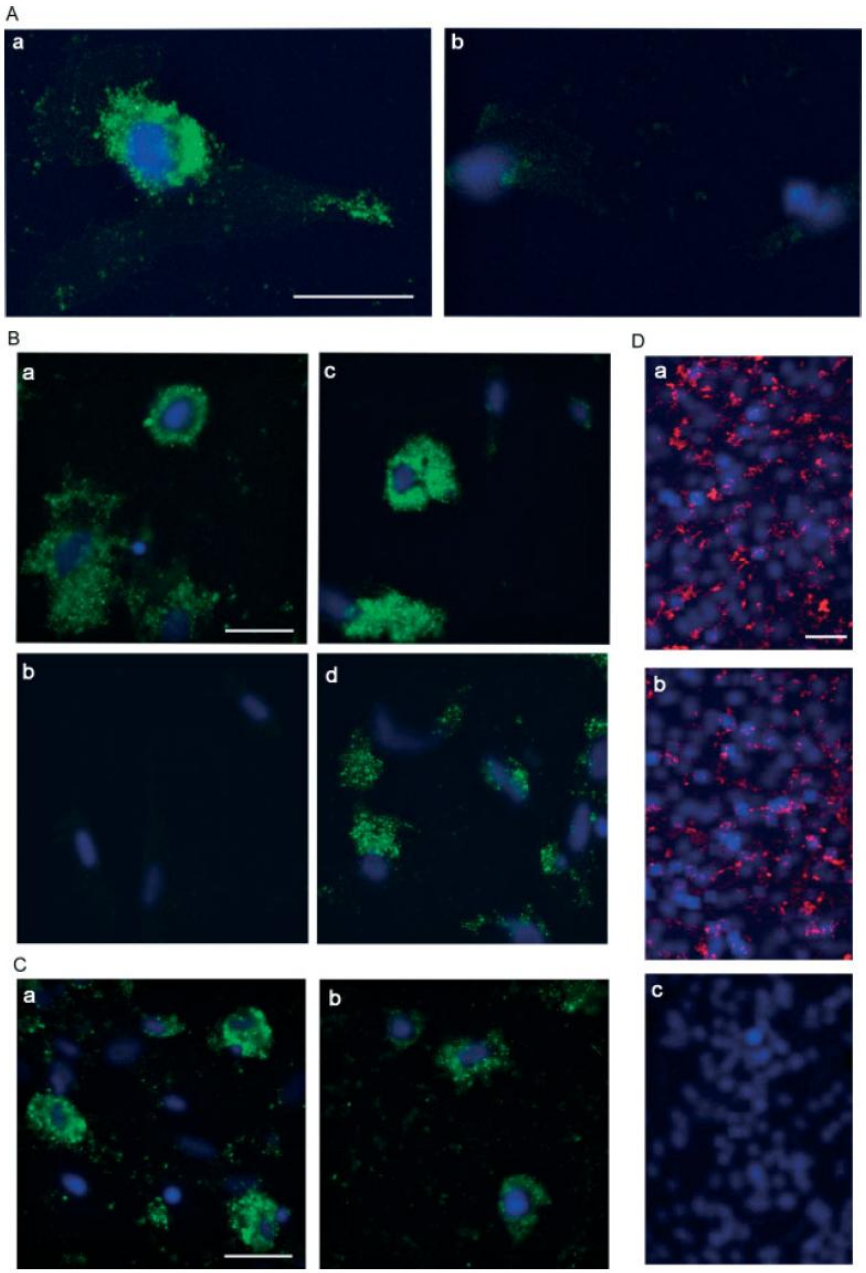

Figure 3. (A) Morphology of PrP immunostaining in variant CJD exposed hESCs. hESCs of the RCM-1 cell line (*PRNP* codon 129-MM genotype) were exposed to variant CJD (a), or grown in control medium (b) for 24 h. hESC cultures were then fixed, permeabilized and immunostained for PrP with the antibody 8H4 (green) and the nuclei counterstained with DAPI (blue). (B) Confirmation of CJD and BSE brain PrP^{Sc} uptake by hESCs, using a modified immunocytochemistry method. hESCs of the RCM-1 cell line were exposed to BSE (a, b) or variant CJD (c, d) 1% brain homogenate for 48 h and then fixed, permiabilized, treated with proteinase K and guanidine and immunostained for PrP with either the 6H4 (a, c) or 3F4 antibody (b, d) (green) and the nuclei counterstained with DAPI (blue). (C) Sporadic CJD brain PrP^{Sc} uptake by hESCs using a modified immunocytochemistry method. hESCs of the RCM-1 cell line were exposed to sporadic CJD MM1 subtype (a) or VV2 subtype (b) 1% brain homogenate for 48 h and then fixed, permiabilized, treated with proteinase K and guanidine and immunostained for PrP with the 6H4 antibody (green) and the nuclei counterstained with DAPI (blue). (D) hESCs of the RCM-1 cell line were exposed to variant CJD (a) or non-CJD (Alzheimer's disease) (b) 1% brain homogenate, or grown in control medium 24 h (c). hESC cultures were then fixed, permeabilized, treated with proteinase K and immunostained with an antibody for glial fibrillary acidic protein (GFAP; red), and the nuclei counterstained with DAPI (blue). Scale bars = 50 μm.

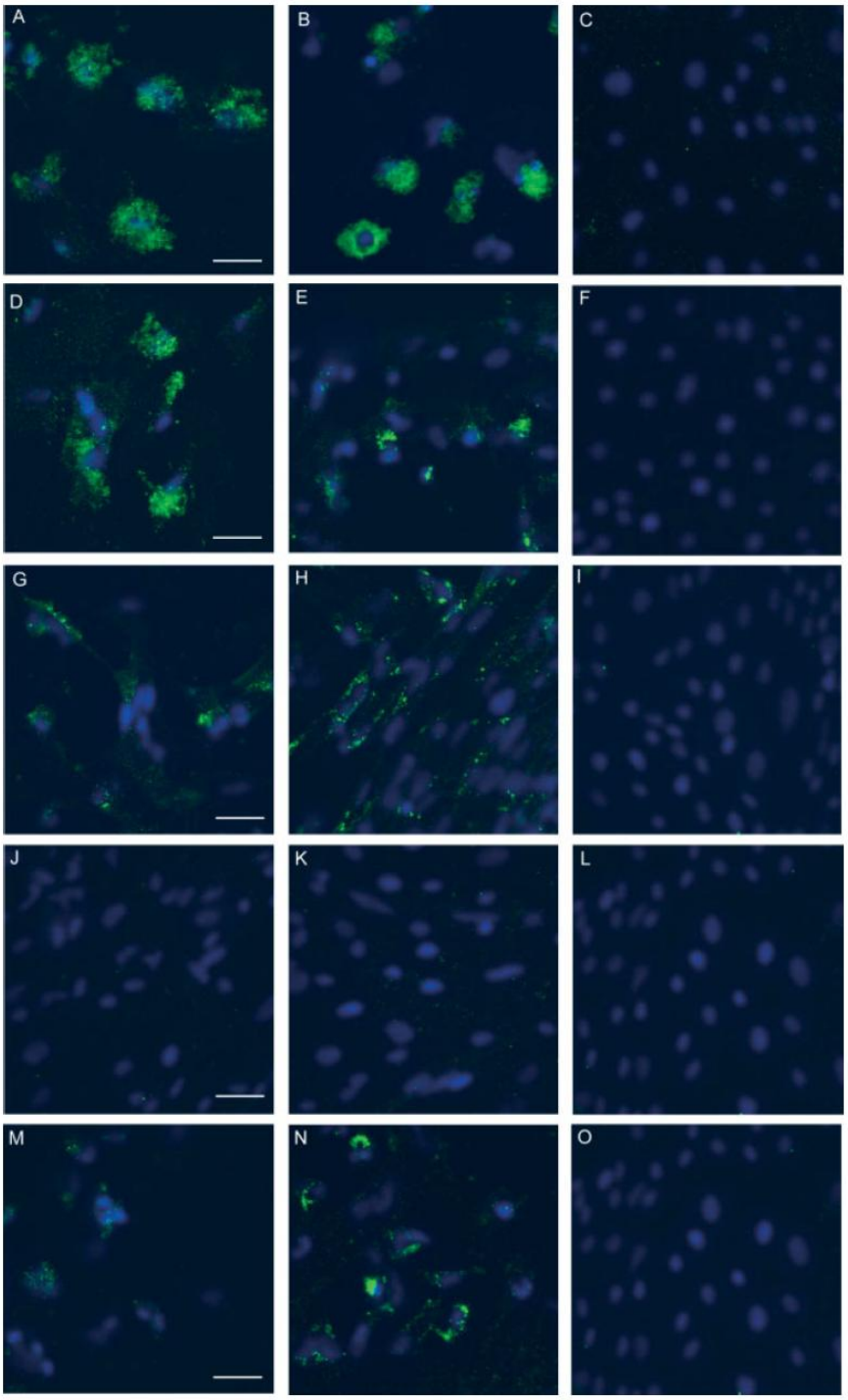

Figure 4. Clearance of PrP^{Sc} from exposed hESCs by immunocytochemistry. hESCs of the RCM-1 cell line (*PRNP* codon 129-MM genotype) were exposed to BSE (A, D, G, J, M), variant CJD (B, E, H, K, N) or Alzheimer's disease (C, F, I, L, O) 1% brain homogenate for 48 h (A–C). The medium was then withdrawn and cells were given fresh medium (without brain homogenate) and allowed to continue growing for a further 24 h (D–F), 48 h (G–I) or 72 h (J–O) with medium changes at 24 h intervals. hESC cultures were fixed, permeabilized, treated with guanidine and proteinase K and immunostained for PrP using the antibody 6H4 (green). The nuclei were counterstained with DAPI (blue). The fields shown in (J–L) are typical of the cultures at the 72 h time point. Infrequent clusters of cells immunostaining for PrP^{Sc} at the 72 h time point are also shown in (M, N). Scale bars (shown in left hand column only) = 50 μm.

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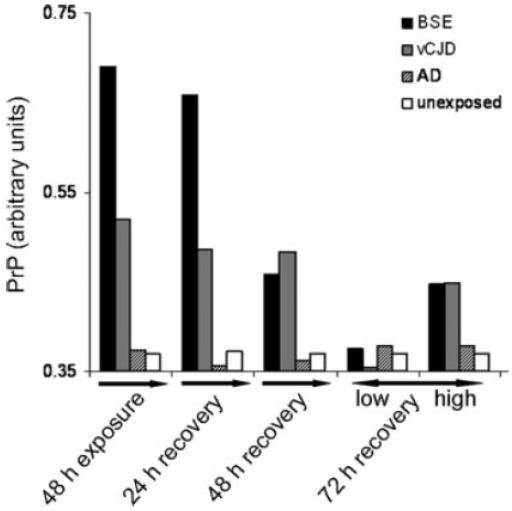

Figure 5. Semi-quantitative assessment of PrP uptake and loss from hESCs of the RCM-1 cell line (*PRNP* codon 129-MM genotype) exposed to BSE, variant CJD (vCJD) and Alzheimer's disease (AD) 1% brain homogenate for 48 h, followed by 24, 48 and 72 h recovery. Unexposed cultures were used as a measure of background values. The green (PrP) and blue (DAPI) fluorescence in the micrographs shown in Figure 4 were determined as activated pixels in Adobe Photoshop® and the green fluorescence was divided by the blue fluorescence to give a measure of PrP staining normalized by cell number. Semi-quantitative assessments were made on both the typical fields (low) and the more rare PrP^{Sc} positive clusters of cells (high).

in neurons where its expression level is high, has been the subject of considerable interest, speculation and competing claims [37,38]. PrP^{C} expression is not, however restricted to the nervous system. It is also found in a wide variety of tissues and cell types, where its function need not reflect that of neural cells. PrP^{C} is associated with the long-term repopulating function of haematopoetic stem cells [39,40] and has recently been suggested to play a role in regulating renewal versus differentiation in hESC cultures [41]. Our data are consistent with a pleiotropic role for PrP^{C} in stem cells during development and differentiation, as previously suggested [42], including involvement in both long-term self-renewal of the stem cell populations and in differentiated cell-associated functions.

Much attention has recently focused on the prevention of secondary spread of variant CJD through iatrogenic means. Variant CJD has thus far only affected patients of the *PRNP* codon 129-MM genotype, although a possible case of variant CJD has recently occurred in a heterozygous patient [43]. Clinical secondary variant CJD (acquired by blood transfusion) has similarly occurred only in 129-MM individuals [14], but evidence of peripheral infection has also been found in two individuals of the 129-MV genotype, exposed to variant CJD-implicated blood or blood products [16,44]. It has been predicted on the basis of animal models that the 129-MM and 129-MV genotypes are equally susceptible to variant CJD transmission [45]. We therefore chose to examine one 129-MM (RCM-1) and one 129-MV (RH1) hESC line to further investigate in terms of their response to exposure to infectious brain material.

These results show that hESCs have mechanisms that allow for the rapid uptake of brain material. This includes the disease-associated and probably infectious form of the prion protein, as judged by the sensitive and specific immunostaining of PrP^{Sc} in exposed cells and the loss of protease-resistant prion protein from the brain-spiked medium, as shown by western blotting. The mechanisms involved in uptake do not appear specific, in that they apply to bovine and human prion disease brain homogenates from patients with either variant or sporadic CJD of the two most frequently occurring subtypes (MM1 and VV2), and occur in both the 129-MM and the 129-MV *PRNP* codon 129 hESCs. The non-specific uptake of PrP^{Sc} by hESCs observed here is fully consistent with previous observations of the uptake of murine-adapted sheep scrapie brain PrP^{Sc} by murine neuronal and fibroblast cell lines [46]. The material taken up by hESCs is not solely PrP but is likely to be a complex mixture of brain components. Moreover, the cells appear to be able to rapidly clear this material when transferred to fresh medium. The rate at which this occurs makes it unlikely that the material is simply diluted out by cell division (a doubling time of 35 h is typical for hESCs), suggesting instead that hESCs have efficient mechanisms to degrade, or otherwise process, the material taken up, including PrP^{Sc}. The finding that exposure of hESCs to high levels of PrP^{Sc} does not result in gross observable cytotoxicity is, in itself, an interesting finding. Whether or not this results from the nature of the cells (ie their non-neuronal phenotype), the fact that the PrP^{Sc} is exogenous rather than produced *in situ*, or whether its effects are circumscribed by its subcellular localization will require further investigation. However, understanding the mechanisms by which cells such as these clear PrP^{Sc} could provide valuable insights into the nature of cellular resistance to prion infection and to the possible causes of sporadic forms of the disease, in which normal clearance mechanisms might have failed [47].

These findings are provocative because, although the hESCs take up and rapidly process PrP^{Sc}, the potential longer-term effects of such exposure are not known. Whether all cells are able to clear PrP^{Sc} completely, and whether they do so before a prion infection can be established remains to be shown, as does the consequence of cellular differentiation of hESCs previously exposed to prions. Differentiated murine neural stem cells, murine neurospheres and differentiated murine bone marrow stromal cells all support the replication of mouse adapted prions [1,48,49]. The low level of PrP^{C} on the surface of hESCs might in itself be sufficient to minimize the chances of hESC infection *in vitro* [50]. However, clinical applications of hESCs will involve

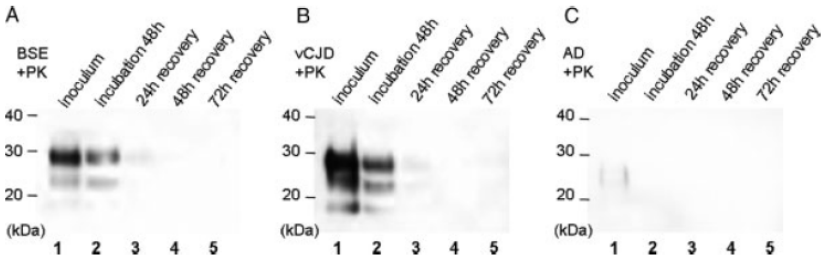

Figure 6. Western blot analysis of protease-resistant prion protein in culture medium used to expose RCM-1 cell line hESCs (*PRNP* codon 129-MM) to BSE (A), variant CJD (B) and non-CJD (Alzheimer's disease) (C). Equivalent volumes of hESC cell culture medium were digested with proteinase K and analysed by western blotting, using the antibody 6H4. The culture media analysed were: (1) the 1% brain inoculum in culture medium prior to incubation with the cells; (2) the same culture medium after 48 h incubation with the cells; (3) fresh medium after a further 24 h of incubation; (4) the second change of fresh medium after a further 24 h of incubation (48 h recovery); and (5) the third change of fresh medium after a further 24 h of incubation (72 h recovery).

cells that have undergone differentiation *in vitro* prior to transplantation. Differentiation-associated changes in the PrP expression may render hESCs susceptible to infection from media components or may allow low level contamination to become an established infection.

It might be argued that the likelihood of animal or human prion contamination of hESCs is remote; nevertheless, the consequences of any such event, however unlikely, could be very serious indeed when one considers the numbers of individuals who might receive cellular therapies from individual cell lines. Given these concerns and our incomplete knowledge of the potential risks posed by known and emergent human and animal prion diseases world-wide, two strategies to assess and manage these risks could be proposed: first, further research on the potential of hESCs to harbour and propagate this novel class of infectious pathogen; and second, renewed efforts to develop methods of hESC derivation and cultivation that minimize exposure to potential sources of human and animal prions.

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Author contributions

SP and PF performed the cell culture and gene expression studies. KS and GRB performed the flow cytometry experiments. The uptake, immunofluorescence and confocal imaging studies were performed by ZK and EC. Genetics studies were performed by MB and characterized human pathology specimens were provided by JWI. The study was conceived, directed and interpreted by PDeS, MLT, JCM and MWH. The manuscript was drafted by MWH with contributions and approval by all authors.

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